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RESEARCH ARTICLE

# Optimization of submerged fermentation conditions for glucanase production by *Burkholderia pyrrocinia* B1213 using *Jiuzao*

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## ABSTRACT

This study optimized the fermentation conditions for producing glucanase from *Burkholderia pyrrocinia* B1213 using *Jiuzao*, a residue from *Baijiu* distillation, as the carbon source. The effects of *Jiuzao* particle size and concentration, type of nitrogen source, urea concentration, initial pH, inoculum size, loading volume, shaking speed, temperature, surfactant type and incubation time on glucanase production by *B. pyrrocinia* B1213 were investigated separately through single factor design. Then, five variables, *Jiuzao* concentration, inoculum size, initial pH, temperature and incubation time, were found to significantly affect glucanase production using the Plackett-Burman design. Following, the optimal conditions for glucanase production by *B. pyrrocinia* B1213 were found using the steepest ascent path and response surface methodology designs as: particle size, 40-60 mesh; *Jiuzao* concentration, 58.4 g/L; urea concentration, 8 g/L; initial pH, 6; loading volume, 15 mL/250 mL; inoculum size, 0.63% (v/v); temperature, 26 °C; shaking speed, 160 rpm; and incubation time, 120 h. Under these conditions, the glucanase activity of the *B. pyrrcinia* B1213 strain was 1336 U/mL, producing the biological enzymes needed in *Baijiu* making from a by-product. This study has provided experimental data and theoretical information for using *B. pyrrcinia* B1213 in *Baijiu* production.

**Keywords:** *Burkholderia pyrrocinia*; Glucanase; Response surface methodology; Fermentation conditions; *Jiuzao*

## INTRODUCTION

Glucanase is widely used as an important industrial enzyme to reduce the adverse effects of glucan in cereal crop processing by degrading the β-glucosidic bond of glucan in cereals. Glucanase plays an active role in the feed, food, textile and paper industries. Many examples can be proposed: in animal feed, the digestibility and growth rate of animals can be improved by glucanase because it improves feed utilization (Duarte et al., 2021; Toghyani et al., 2022). Glucanase can also improve filtration efficiency in the beer brewing process (Guo et al., 2010); it can smoothen the surface of fabrics so that they feel thick and soft (Sahin et al., 2016); and in paper production, glucanase promotes the absorption, swelling and fibrillation of fibers, improves pulping performance, reduces the energy consumed by pulping, improves paper quality, and plays an important role in the biological deinking of paper (Biswas et al., 2019). Glucanase is derived from many sources, such as animals, plants and microorganisms. Of these, microorganisms are the main source of the enzyme because of the short fermentation cycle, high production capacity and easy industrial production process. Several microorganisms such as bacteria, mycobacteria, actinomycetes and yeasts from different habitats have been screened to assess their ability to produce glucanase, thus providing alternative resources for industrial application (Fayad et al., 2001; Qiao et al., 2010; van Rensburg et al., 1997; Wang et al., 2007). As mining functions for identifying and quantifying *in situ* microorganisms in specific habitats have been developed and their applications have expanded, more studies have focused on the functions of *in situ* microorganisms and their intrinsic mechanisms. This will provide important information for improving the applications of microorganisms in their original ecology, and also new directions for enzyme resource mining and

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function development (Du et al., 2019; Jung et al., 2021; Jung et al., 2016; Van Nostrand et al., 2011).

*Baijiu*, which occupies a vital position in China's food industry and its total sales revenue reached 583.639 billion yuan in 2020, is made by a complex fermentation process using natural mixed-culture starters (Fan et al., 2021; Wang et al., 2022). The environment for *Baijiu* brewing is a very complex habitat, with a large number of habitat-specific microorganisms and a certain regular repetitive microbial turnover. The development of these microbial functions will contribute to the scientific and standardization of *Baijiu* brewing in China (Wang, 2022), thereby improving product quality. At present, many microorganisms from specific brewing environments have been discovered and identified, allowing a bank of microbial strains to be established for *Baijiu* brewing. During these studies, many novel microorganisms and enzymes with excellent functional properties have been discovered (Ali et al., 2019; Fan et al., 2020; Fan et al., 2021; Lu et al., 2021; Wang et al., 2020). One strain, *Burkholderia pyrrocinia* B1213 with high lipase production, was obtained from the *Baijiu* brewing environment during earlier research (Li et al., 2018). Genomic analysis has highlighted its potential applications for producing esters in *Baijiu* brewing, and for degrading lignocellulose (Hu et al., 2020). The activity of glucanase produced by *B. pyrrocinia* B1213 in a medium with wheat bran as the carbon source was more than 1200 U/mL at an optimum temperature which was consistent with the ambient temperature of *Baijiu* brewing. The consequent hydrolysis of glucan mainly produced cellotriose with cellotetrose and cellopentaose (Hu, 2020). These characteristics suggest that *B. pyrrocinia* B1213 provide more potent applications in *Baijiu* brewing, such as improving the degradation of cellulose in sorghum to release starch leading to an improvement in efficiency and yield and increasing the added value of *Jiuzao*, a by-product of *Baijiu* production, by producing glucanase and cello-oligosaccharides (Guo and Jia, 2015; Hu, 2020). Nearly 100 million tons of *Jiuzao* are generated each year from the process of *Baijiu* brewing and are usually discarded, although it has rich nutrient contents, and is thus an excellent natural medium for the growth and reproduction of many microorganisms, creating an environmental concern since their decay produces a foul odor (Qin et al., 2022). It has been reported to enhance glucanase production by microorganisms because it is rich in fibrous components (Jiang et al., 2022). The activity of glucanase, obtained by *B. pyrrocinia* B1213 in a medium with *Jiuzao* as the carbon source, was lower than a medium reported earlier as the best carbon source using wheat bran (Wei et al., 2023). Therefore, the present study investigated the production of glucanase by *B. pyrrocinia* B1213 with *Jiuzao* as the carbon source. The results of this study will help to explore the *in situ* application of *Burkholderia* sp. during the *Baijiu* brewing process, thus increasing the utilization of *Baijiu* brewing waste and reducing environmental pollution and also the waste of resources. The study will also provide experimental data for the *Baijiu* brewing industry that can be used as a reference for carbon peaking and carbon neutralization.

## MATERIALS AND METHODS

### Strain and reagents

*B. pyrrocinia* B1213 was collected from *Baijiu* brewing environment and preserved in our laboratory. Barley glucan and glucose standard were purchased from Sigma. *Jiuzao* were provided by Chengde Bancheng Liquor Sales Co., Ltd. All other reagents used were domestic biological or analytical grade reagents unless otherwise stated.

### Medium

Luria-Bertani (LB) medium was prepared with 10 g tryptone, 5 g yeast extract powder, 10 g sodium chloride and 1000 mL dd$H_2O$, natural pH, autoclaved at 115 °C for 20 min as previous report (Hu et al., 2020).

Fermentation was conducted in a medium (fermentation medium) composed of 40 g *Jiuzao* (20-40 mesh), 4 g yeast extract powder, 0.37 g $KH_2PO_4$, 0.07 g $CaCl_2{\cdot}2H_2O$, 0.25 g $MgSO_4{\cdot}7H_2O$, 0.02 g $FeCl_3$ and 1000 mL dd$H_2O$ with pH 7.0 and autoclaved at 115 °C for 20 min to produce glucanase as our previous study (Wei et al., 2023).

### Optimization of glucanase production conditions by a single factor design

*B. pyrrocinia* B1213 was activated by inoculation into LB medium at 30 °C and shaken at 120 rpm for 12 h. After twice activation, 0.5% (v/v) inoculum were cultivated into 250 mL Erlenmeyer flask containing 30 mL fermentation media at 30 °C, 120 rpm for 96 h as our previous study (Wei et al., 2023). The culture was then centrifuged at 4°C, 10,000 rpm for 10 min to obtain crude enzyme. Various culture conditions (Supplementary Table S1), including *Jiuzao* particle size, *Jiuzao* concentration, nitrogen source type, urea concentration, initial pH, inoculum size, loading volume, shaking speed, temperature, surfactant type and time, were optimized for glucanase production by using a single factor design under submerged fermentation.

### Optimization of glucanase production conditions by Plackett-Burman (PB) design

According to the results from the single factor design, seven factors, including *Jiuzao* concentration, urea concentration, inoculum size, shaking speed, initial pH, temperature and time, were selected for the PB design at two levels to determine their effect on glucanase production, and the other factors were set at the best conditions in the single

factor design (Table 1) according to previous report (Yang et al., 2021).

### Optimization of glucanase production conditions by steepest ascent path design

Five factors with significant effect on glucanase activity were obtained from the PB design to construct the steepest ascent path design according to Hu et al (2020). The direction and step length of the factors in the steepest ascent path design were determined as regression results of PB design and practical experience. Five experiments were conducted as other factors with no significant effect on glucanase production at the best conditions in single factor design (Table 2).

### Optimization of glucanase production conditions by response surface methodology (RSM)

RSM was used to further optimize the first three factors (inoculum size, time and *Jiuzao* concentration) identified by PB design that have the most significant impact on glucanase production. The center point of RSM was the point where the glucanase production was highest in the steepest ascent path design. Fifteen test groups, including three replicates at the center point, were designed using the Box-Behnken experimental design (BBD, Design-Expert Software 11.0) according to the method reported by Yang et al (as in Table 3) (2021). The initial pH and temperature were set at the values corresponding to the highest enzyme activity group in the steepest ascent path design, respectively.

### Glucanase activity assay

Glucanase activity was determined according to our previous reported (Hu et al., 2020). The specific steps were as follows: 25 μL of a suitably diluted enzyme solution with 50 mM phosphate buffer solution of pH 6.0 was mixed with 225 μL of 1% (w/v) barley glucan, then, was incubated at 45 °C for 10 min. After reaction, 250 μL of 3,5-dinitrosalicylic acid reagent was added and the mixture was boiled for 15 min. Then, 250 μL of 40% potassium sodium tartrate solution was added after cooling, and the absorbance was measured at a wavelength of 540 nm. The amount of reducing sugar released was calculated using glucose as standard. One unit (U) of glucanase activity was defined as the amount of enzyme required to produce 1 μmol of glucose in 1 min under the above assay conditions.

**Table 1: Factors and levels of the variables in PB design for glucanase production**

| Factors | Level | |
|---|---|---|
| | -1 | +1 |
| $X_1$-*Jiuzao* concentration (g/L) | 20 | 60 |
| $X_2$-Urea concentration (g/L) | 4 | 12 |
| $X_3$-Inoculum size (%, v/v) | 0.5 | 1.5 |
| $X_4$-Shaking speed (rpm) | 120 | 200 |
| $X_5$-Initial pH | 5 | 9 |
| $X_6$-Temperature (°C) | 25 | 35 |
| $X_7$-Time (h) | 72 | 120 |

### Statistical analysis

Assays have been conducted in triplicate, and the reported values correspond to the mean value. Statistical differences among treatment groups were analyzed using a one-way ANOVA ($p<0.05$) followed by Tukey test. SPSS 28.0 (IBM Corp., New York, USA), Design-Expert 11.0 (State-Ease, Inc. USA) and Excel 2019 (Microsoft, USA) were used to analyze the data.

## RESULTS AND DISCUSSION

### Optimization of production conditions using a single factor design

*Effect of Jiuzao particle size on glucanase production*

The particle size of insoluble nutrients affects their dispersion in a matrix, which in turn affects their degree of decomposition and utilization efficiency by microorganisms, thus influencing microbial growth, reproduction, and metabolites. Therefore, the effect of the *Jiuzao* particle size on glucanase production by *B. pyrrocinia* B1213 was studied. The results showed that the glucanase activity of *B. pyrrocinia* B1213 increased then decreased as the *Jiuzao* particle size decreased (Fig. 1). The highest activity was obtained with a particle size of 40-60 mesh. This was consistent with previous studies where the highest activity was obtained when the particle size of insoluble nutrients was approximately 20-80 mesh. If the particle size was too great, the specific surface area was small, so that *B. pyrrocinia* B1213 could not utilize the nutrients in the *Jiuzao*, and if too small, the specific surface area increased so that *B. pyrrocinia* B1213 could rapidly obtain nutrients from

**Table 2: Experimental designs and the results of the steepest ascent path design for glucanase production**

| Number of tests | *Jiuzao* concentration (g/L) | Inoculum size (%, v/v) | Initial pH | Temperature (°C) | Time (h) | Glucanase activity (U/mL) |
|---|---|---|---|---|---|---|
| 1 | 20 | 1.5 | 9 | 35 | 144 | 726 |
| 2 | 30 | 1.25 | 8 | 32 | 126 | 985 |
| 3 | 40 | 1 | 7 | 29 | 108 | 1044 |
| 4 | 50 | 0.75 | 6 | 26 | 90 | 1206 |
| 5 | 60 | 0.5 | 5 | 23 | 72 | 675 |

insoluble nutrients then grow and reproduce abundantly. This caused the viscosity of the medium to increase and a low level of dissolved oxygen during the fermentation process, leading to a low enzyme production level (Chandra et al., 2010; Shah et al., 2015).

### *Effect of Jiuzao concentration on glucanase production*

*Jiuzao* is rich in nutrients, such as cellulose, hemicellulose, protein and vitamins and therefore an important carbon source for microbial growth and reproduction, and for encouraging microorganisms to produce a series of hydrolytic enzymes for breaking down fiber (Ning et al., 2021). Therefore, the *Jiuzao* concentration influences glucanase production using *B. pyrrocinia* B1213. The glucanase production was low when the concentration was low because the carbon sources available were insufficient for the growth of *B. pyrrocinia* B1213 during fermentation to produce glucanase. As the *Jiuzao* concentration increased, the glucanase activity increased to 420 U/mL when the *Jiuzao* concentration was 60 g/L. The glucanase activity decreased when the *Jiuzao* concentration exceeded 60 g/L because of the increase in the medium viscosity, the reduction in the level of dissolved oxygen and the increased amounts of some harmful substances such as weak acids (Fig. 2). To induce most microorganisms to produce enzymes, the optimal concentration range for agricultural and forestry by-products for use as a carbon source has been reported as 40-70 g/L. In this concentration range, using agricultural and forestry by-products as carbon sources can meet the needs of microbial growth and reproduction for producing enzymes, and ensure a suitable dissolved oxygen concentration for microbial metabolic activities (Cao et al., 2008; Kim et al., 2007; Tang et al., 2004; Yardimci and Cekmecelioglu, 2018).

**Table 3: Factors and levels for the Box-Behnken design**

| Factors | Level | |
|---|---|---|
| | -1 | +1 |
| A-Inoculum size (%, v/v) | 0.50 | 1.00 |
| B-Time (h) | 72 | 120 |
| C-*Jiuzao* concentration (g/L) | 40 | 60 |

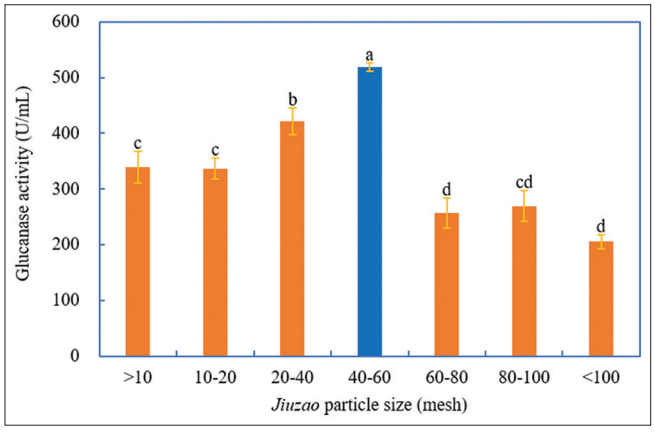


**Fig 1.** Effect of *Jiuzao* particle size on the glucanase production by *B. pyrrocinia* B1213. Values represented by columns with the same letter do not differ significantly at 5% probability by the Tukey test.

### Effect of nitrogen source type and its concentration on glucanase production

Most studies have shown that the best type of nitrogen source for producing the same metabolites varies according to the microbial strain or for the same strain to produce different metabolites (Rani et al., 2014). Therefore, the most suitable type of nitrogen source needed to be selected and optimized to obtain microbial metabolites economically and efficiently (Hungaro et al., 2013; Rani et al., 2014). In general, the amounts of metabolites produced by microorganisms using organic sources of nitrogen are greater than by using inorganic sources of nitrogen (Ramirez-Lagunes et al., 2021). In the present study, except for tryptone, the production of glucanase by *B. pyrrocinia* B1213 with different nitrogen sources of the same effective nitrogen content did not differ significantly, possibly because a few of protein and amino acids in *Jiuzao* provided a partial nitrogen source (Fig. 3) (Jiang et al., 2022). The highest glucanase activity was obtained using urea as a nitrogen source, whereas the optimal nitrogen source for lipase production by *B. pyrrocinia* B1213 was different and the lowest lipase activity occurred with urea. This result confirmed that different enzymes produced by the same strain required different types of nitrogen source. This may have been caused by differences in the synthesis pathway between glucanase and lipase production by *B. pyrrocinia* B1213, with the differences in the composition of the initial fermentation medium another possible reason. In the present study, the composition of *Jiuzao* was found to be complex, apart from some of the nutrients required for *B. pyrrocinia* B1213 to grow and ferment, it contained a small amount of organic acids which would have affected its growth (Boncz et al., 2012; Li et al., 2018). Urea was better than other nitrogen sources for producing glucanase

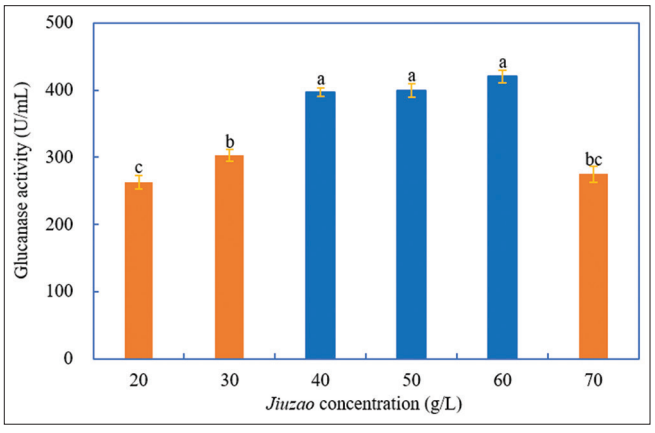


**Fig 2.** Effect of *Jiuzao* concentration on the glucanase production by *B. pyrrocinia* B1213. Values represented by columns with the same letter do not differ significantly at 5% probability by the Tukey test.

from *B. pyrrocinia* B1213 with *Jiuzao* because it could produce ammonia which neutralized the residual organic acids in *Jiuzao* and reduced the negative effects of organic acids on microbial growth and enzyme production. The effect of urea concentration on the production of glucanase was then investigated, and showed that the glucanase activity was relatively high even when the urea concentration was low mainly because of some protein in the *Jiuzao*. The highest activity was obtained when the urea concentration was 8.0 g/L and provided a suitable level of carbon and nitrogen. The secondary metabolism for producing glucanase would be inhibited at a urea concentration over 8.0 g/L because of the low levels of carbon and nitrogen causing vigorous growth (Jiang et al., 2022; Yang et al., 2006).

### Effect of initial pH on glucanase production

The pH of the microbial growth environment would affect cell membrane permeability, and the activity of proteins and enzymes in the cell membrane, as well as the ionization state of nutrients. This therefore changes the capacity of the cell membrane to absorb nutrients under different environmental pH conditions (Ajijolakewu et al., 2016; Gupta et al., 2003). Fig. 5 shows that an initial pH

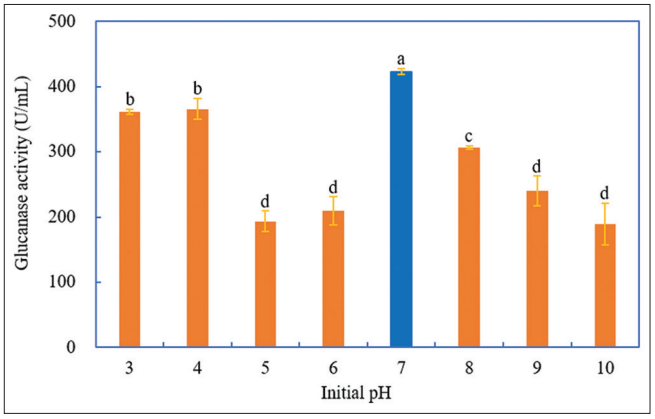


**Fig 5.** Effect of initial pH on the glucanase production by *B. pyrrocinia* B1213. Values represented by columns with the same letter do not differ significantly at 5% probability by the Tukey test.

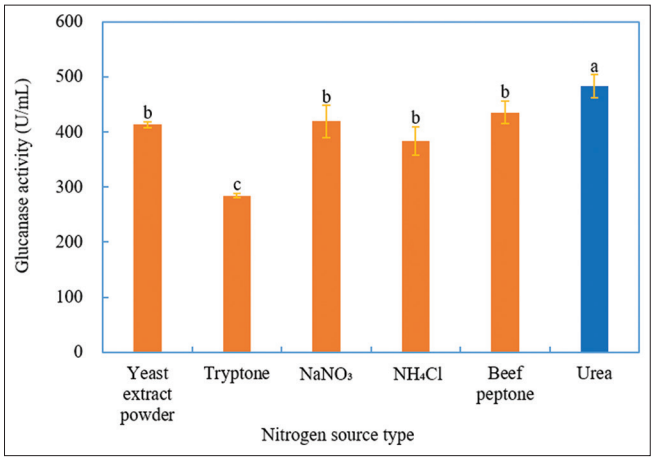


**Fig 3.** Effect of different nitrogen sources on the glucanase production by *B. pyrrocinia* B1213. Values represented by columns with the same letter do not differ significantly at 5% probability by the Tukey test.

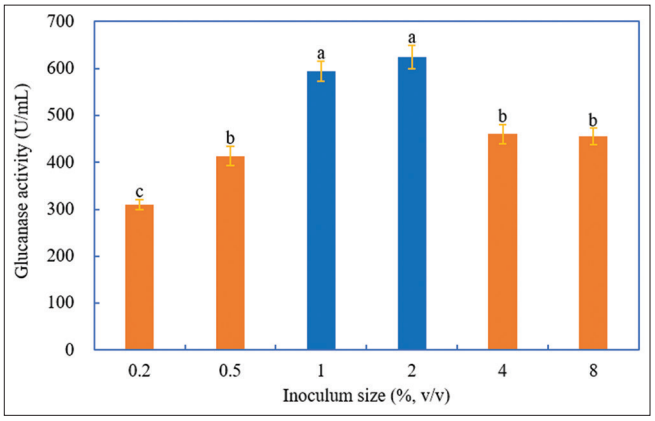


**Fig 6.** Effect of inoculum size on the glucanase production by *B. pyrrocinia* B1213. Values represented by columns with the same letter do not differ significantly at 5% probability by the Tukey test.

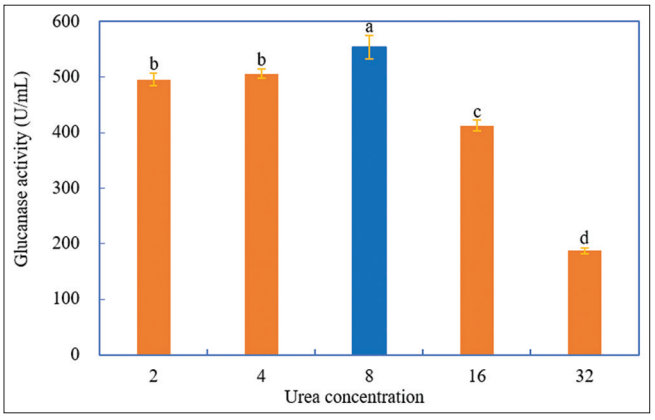


**Fig 4.** Effect of urea concentration on the glucanase production by *B. pyrrocinia* B1213. Values represented by columns with the same letter do not differ significantly at 5% probability by the Tukey test.

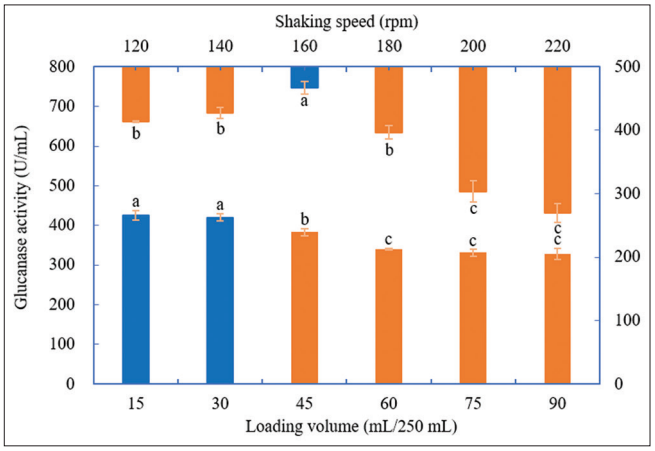


**Fig 7.** Effect of loading volume and shaking speed on the glucanase production by *B. pyrrocinia* B1213. Values represented by columns with the same letter do not differ significantly at 5% probability by the Tukey test.

of 7.0 was most suitable for *B. pyrrocinia* B1213 to produce glucanase, which was consistent with its optimal growth pH. The glucanase activity was also relatively high at a pH of 3-4 because of the diverse components in *Jiuzao*, some of which would change their ionic state to facilitate their utilization by *B. pyrrocinia* in these pH ranges.

### Effect of inoculum size on glucanase production

An appropriate inoculum size can improve enzyme activity and production efficiency. The glucanase activity produced by *B. pyrrocinia* B1213 increased as the inoculum size increased but decreased when the inoculum size exceeded 2%. This agreed with most previous reports, where the optimal inoculum size for bacteria was generally in the range of 1% to 5% (Rajendran et al., 2008). A lower inoculum size often causes a longer growth delay period, which also delays enzyme synthesis. However, a high inoculum size could shorten the growth delay period, but rapid overgrowth would cause competition between nutrients, accelerating the decline in cells and reducing the enzyme production period (Ajijolakewu et al., 2017; Fang et al., 2010; Pathak et al., 2014).

### Effect of loading volume and shaking speed on glucanase production

Dissolved oxygen affects the physiological state of microorganisms through respiration, which influences cell growth, protein secretion and product synthesis (Fuzi et al., 2014; Trentmann et al., 2004). In the present study, the dissolved oxygen level was influenced by the loading volume and the shaking speed during the liquid state fermentation in the shaken flask. A high loading volume and low shaking speed led to a low dissolved oxygen level and *vice versa*. A high dissolved oxygen level promoted high glucanase production by *B. pyrrocinia* B1213 at a low loading volume or at a high shaking speed, which supported the speculation for optimizing the *Jiuzao* particle size conditions. The influence of shear force on the microbial cells should also be taken into account when setting the shaking speed conditions. At a higher shaking speed, although the level of dissolved oxygen was more than sufficient for *B. pyrrocinia* B1213, the shear force caused by the violent collisions between the cells and the *Jiuzao* particles or by shaking the flask was not conducive to the normal growth of *B. pyrrocinia* B1213, leading to a reduction in glucanase activity.

### Effect of temperature on glucanase production

Temperature is critical for microbial growth and reproduction, and regulates the production of microbial enzymes and influences the stability of enzymes thus affecting enzyme production (Gupta et al., 2003). The optimal temperature for glucanase production by *B. pyrrocinia* B1213 was 30 °C, which was consistent with its optimal growth temperature, the thermal stability of glucanase and the optimal temperature for lipase production (Fig. 8) (Hu, 2020; Li et al., 2018).

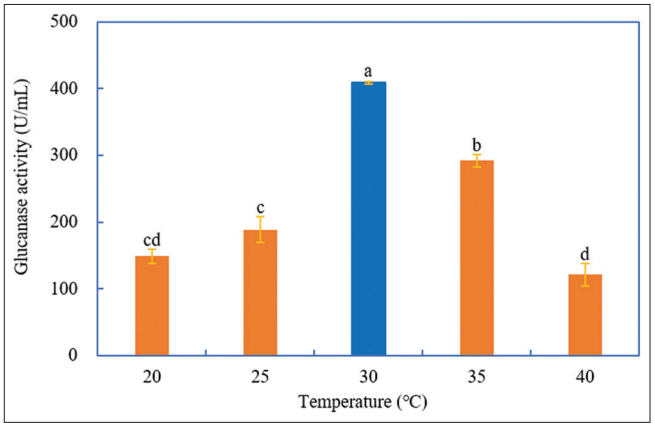


**Fig 8.** Effect of temperature on the glucanase production by *B. pyrrocinia* B1213. Values represented by columns with the same letter do not differ significantly at 5% probability by the Tukey test.

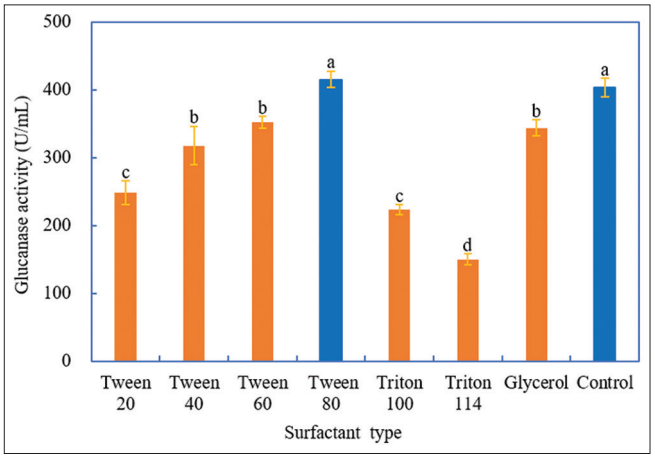


**Fig 9.** Effect of different surfactants on the glucanase production by *B. pyrrocinia* B1213. Values represented by columns with the same letter do not differ significantly at 5% probability by the Tukey test.

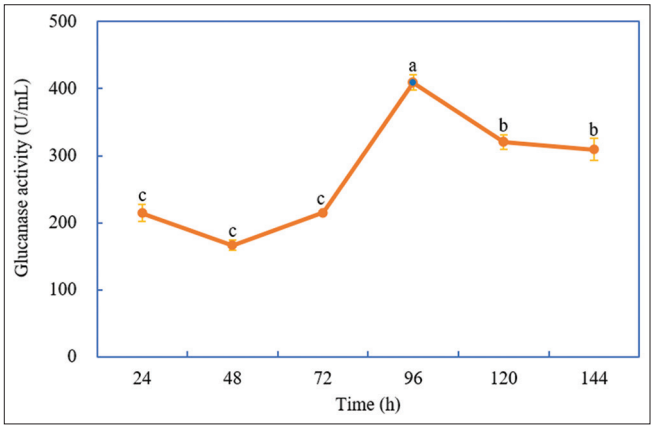


**Fig 10.** Effect of time on the glucanase production by *B. pyrrocinia* B1213. Values with the same letter do not differ significantly at 5% probability by the Tukey test.

*Effect of surfactant type on glucanase production*

Previous reports have shown that the permeability of microbial cell membranes may be altered by adding surfactants, causing a change in nutrient utilization and the secretion of enzymes (Jiang et al., 2005; Ridder et al., 1998). For example, cellulase production by *Melanocarpus* spp. was improved by Tween-20 (Jatinder et al., 2006) and cellulase and xylanase production by *Aspergillus niger* was enhanced by Tween-80 (Kumar et al., 2018). The influence of surfactants on enzyme production varies with the particular microorganism, because of many factors such as microbial cell structure, surfactant type and concentration, culture conditions and the medium composition. Although Tween-80 had no effect on glucanase production by *B. pyrrocinia* B1213, other surfactants tested had certain inhibitory effects, similar to those in lipase production (Li et al., 2018). The cell membrane structure of *B. pyrrocinia* B1213 had possibly been changed greatly by the surfactants, resulting in increased permeability, partial electrolyte extravasation, and metabolic disorders. Some substances in *Jiuzao* that are not conducive to microbial growth would also affect enzyme production with their rapid entry into cells.

**Effect of time on glucanase production**

The growth curves of microorganisms vary, and the optimal time for enzyme production also varies. If the time is too short, enzyme production will be low because of an insufficient number of microbial cells, but if the time is too long, costs will increase and enzyme production may also be low because of degradation by the protease released from dead cells. Therefore, the fermentation time must be optimized to produce enzymes efficiently. The glucanase activity increased then decreased with increasing time, with the highest activity of 409 U/mL at 96 h, similar to lipase production. Both glucanase and lipase are produced mainly during the middle stages of fermentation, particularly the stable stage, because they are induced enzymes, which are secondary metabolites. These results were consistent with the reported growth characteristics of *B. pyrrocinia* (Li et al., 2018).

**Optimization of glucanase production conditions with PB design**

According to results of the single factor design, seven factors including *Jiuzao* concentration ($X_1$), urea concentration ($X_2$), inoculum size ($X_3$), shaking speed ($X_4$), initial pH ($X_5$), temperature ($X_6$) and time ($X_7$) were evaluated for optimization using the PB design, and *Jiuzao* with a particle size of 40-60 mesh was selected as carbon source and urea as nitrogen source. Fifteen runs including three replicates at the center point were designed, and the results for each run were shown in Table 4. Changes in glucanase activity ranged from 445 U/mL to 1012 U/mL, indicating glucanase activity was affected by these factors. A polynomial equation was obtained by regression simulation analysis of the experimental results to explain glucanase production:

$$Y=699.83+58.67X_1-28.67X_2-104.17X_3-0.1667X_4-49.83X_5-51.67X_6-72.83X_7 \quad (1)$$

where Y was the glucanase activity.

The effect of each factor on glucanase production was identified by the Fisher's test and *P* values. The model was highly significant as the *P* value less than 0.01, and the coefficient of determination ($R^2$) was 0.9279, suggesting a high correlation between predicted and experimental values. Based on the coefficient of regression and the statistical confidence level, five factors, inoculum size, time, *Jiuzao* concentration, temperature and initial pH, out of the seven factors examined significantly influenced glucanase activity at the 5% level of significance. And, among them, *Jiuzao* concentration had a positive coefficient, whereas other four factors showed negative coefficient. Thus, *Jiuzao* concentration should be increased and other four factors should be decreased in further study. Since urea concentration and shaking speed had an insignificant effect on glucanase activity, they were omitted from further study and were set to 8 g/L and 160 rpm, respectively, according to the single factor results.

**Optimization of glucanase production conditions with steepest ascent path design**

Based on results of PB design, five tests, which step length of each factor was depended on equation (1) of the step length of the steepest ascent method and experimental experience. Glucanase activity increased along the path and plateaued in test 4 with the highest activity of 1206 U/mL under cultivation for 90 h at pH 6.0, 26 °C, and 50 g/L *Jiuzao* with an inoculum size of 0.75% (v/v) (Table 2). Therefore, the fourth set of tests was used as the central point of subsequent RSM.

**Optimization of glucanase production conditions with RSM design**

Three factors (inoculum size (A), time (B) and *Jiuzao* concentration (C)) that the first three most significant factors to glucanase activity obtained from PB design results and three-level with the central point determined by steepest ascent path design were carried out for RSM design by BBD. A total of 15 experiments, including three replicates at the center point was used to analyze the interactions between the three factors and to obtain the best fermentation conditions (Table 6). The maximum glucanase activity was 1331 U/mL and the minimum was 891 U/mL. A 2nd-order polynomial equation was obtained as the regression model for glucanase production:

**Table 4: PB design matrix for evaluating factors influencing on glucanase production**

| Test number | Factors | | | | | | | Glucanase activity (U/mL) |
|---|---|---|---|---|---|---|---|---|
| | $X_1$-*Jiuzao* concentration (g/L) | $X_2$-Urea concentration (g/L) | $X_3$-Inoculum size (%, v/v) | $X_4$-Shaking speed (rpm) | $X_5$-Initial pH | $X_6$-Tempreture (°C) | $X_7$-Time (h) | |
| 1 | -1 | 1 | 1 | -1 | -1 | -1 | 1 | 590 |
| 2 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1012 |
| 3 | 1 | -1 | 1 | -1 | 1 | -1 | -1 | 823 |
| 4 | -1 | 1 | 1 | 1 | -1 | 1 | -1 | 591 |
| 5 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | 759 |
| 6 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | 532 |
| 7 | 1 | -1 | 1 | 1 | -1 | 1 | 1 | 593 |
| 8 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | 445 |
| 9 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | 798 |
| 10 | -1 | 1 | -1 | -1 | 1 | 1 | 1 | 543 |
| 11 | 1 | -1 | -1 | -1 | -1 | 1 | 1 | 832 |
| 12 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 880 |
| 13 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 880 |
| 14 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 883 |
| 15 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 936 |

**Table 5: Model partial regression coefficient and significance analysis in PB design**

| Model items | Regression coefficient | Degree of freedom | Standard Error | F-value | *P*-value | Significant |
|---|---|---|---|---|---|---|
| Models | $3.069\times10^5$ | 7 | 43837.33 | 11.04 | 0.0047 | ** |
| *Jiuzao* concentration | 41301.33 | 1 | 41301.33 | 10.40 | 0.0180 | * |
| Urea concentration | 9861.33 | 1 | 9861.33 | 2.48 | 0.1662 | |
| Inoculum size | $1.302\times10^5$ | 1 | $1.302\times10^5$ | 32.78 | 0.0012 | ** |
| Shaking speed | 0.3333 | 1 | 0.3333 | 0.0001 | 0.9730 | |
| Initial pH | 29800.33 | 1 | 29800.33 | 7.50 | 0.0338 | * |
| Temperature | 32033.33 | 1 | 32033.33 | 8.06 | 0.0296 | * |
| Time | 63656.33 | 1 | 63656.33 | 16.03 | 0.0071 | ** |
| Residual | 23833.00 | 6 | 3972.17 | | | |
| Lack of Fit | 21848.33 | 4 | 5462.08 | 5.50 | 0.1596 | |
| Pure Error | 1984.67 | 2 | 992.33 | | | |
| Total | $4.265\times10^5$ | 14 | | | | |

**: highly significant difference ($P<0.01$); *: significant difference ($P<0.05$).

**Table 6: The Box-Behnken design and the responses of the dependent variables**

| Standard serial number | Test serial number | A-Inoculum size | B-Time | C-*Jiuzao* concentration | Glucanase activity (U/mL) |
|---|---|---|---|---|---|
| 1 | 1 | 0.5 | 72 | 50 | 1096 |
| 2 | 2 | 1 | 72 | 50 | 1172 |
| 3 | 3 | 0.5 | 120 | 50 | 1266 |
| 4 | 4 | 1 | 120 | 50 | 1054 |
| 5 | 5 | 0.5 | 96 | 40 | 1006 |
| 6 | 6 | 1 | 96 | 40 | 891 |
| 7 | 7 | 0.5 | 96 | 60 | 1205 |
| 8 | 8 | 1 | 96 | 60 | 1123 |
| 9 | 9 | 0.75 | 72 | 40 | 1068 |
| 10 | 10 | 0.75 | 120 | 40 | 943 |
| 11 | 11 | 0.75 | 72 | 60 | 1308 |
| 12 | 12 | 0.75 | 120 | 60 | 1331 |
| 13 | 13 | 0.75 | 96 | 50 | 1291 |
| 14 | 14 | 0.75 | 96 | 50 | 1235 |
| 15 | 15 | 0.75 | 96 | 50 | 1283 |

**Table 7: Regression coefficients and their significances for glucanase production from the results of the Box-Behnken design**

| Source | Sum of squares | Degree of freedom | Mean Square | F-value | *P*-value | Significant |
|---|---|---|---|---|---|---|
| Models | $2.596\times10^5$ | 9 | 28843.15 | 14.26 | 0.0046 | ** |
| A | 13861.13 | 1 | 13861.13 | 6.85 | 0.0472 | * |
| B | 312.50 | 1 | 312.50 | 0.1545 | 0.7105 | |
| C | $1.402\times10^5$ | 1 | $1.402\times10^5$ | 69.29 | 0.0004 | *** |
| AB | 20736.00 | 1 | 20736.00 | 10.25 | 0.0240 | * |
| AC | 272.25 | 1 | 272.25 | 0.1346 | 0.7288 | |
| BC | 5476.00 | 1 | 5476.00 | 2.71 | 0.1608 | |
| $A^2$ | 48371.85 | 1 | 48371.85 | 23.91 | 0.0045 | ** |
| $B^2$ | 248.78 | 1 | 248.78 | 0.1230 | 0.7401 | |
| $C^2$ | 36157.85 | 1 | 36157.85 | 17.87 | 0.0083 | ** |
| Residual | 10115.42 | 5 | 2023.08 | | | |
| Lack of Fit | 8280.75 | 3 | 2760.25 | 3.01 | 0.2593 | |
| Pure Error | 1834.67 | 2 | 917.33 | | | |
| Correlation coefficient | $R^2$=0.9625 | $R^2_{Adj}$=0.8950 | | | | |

***: highly significant difference ($P$<0.001); **: highly significant difference ($P$<0.01); *: significant difference ($P$<0.05).

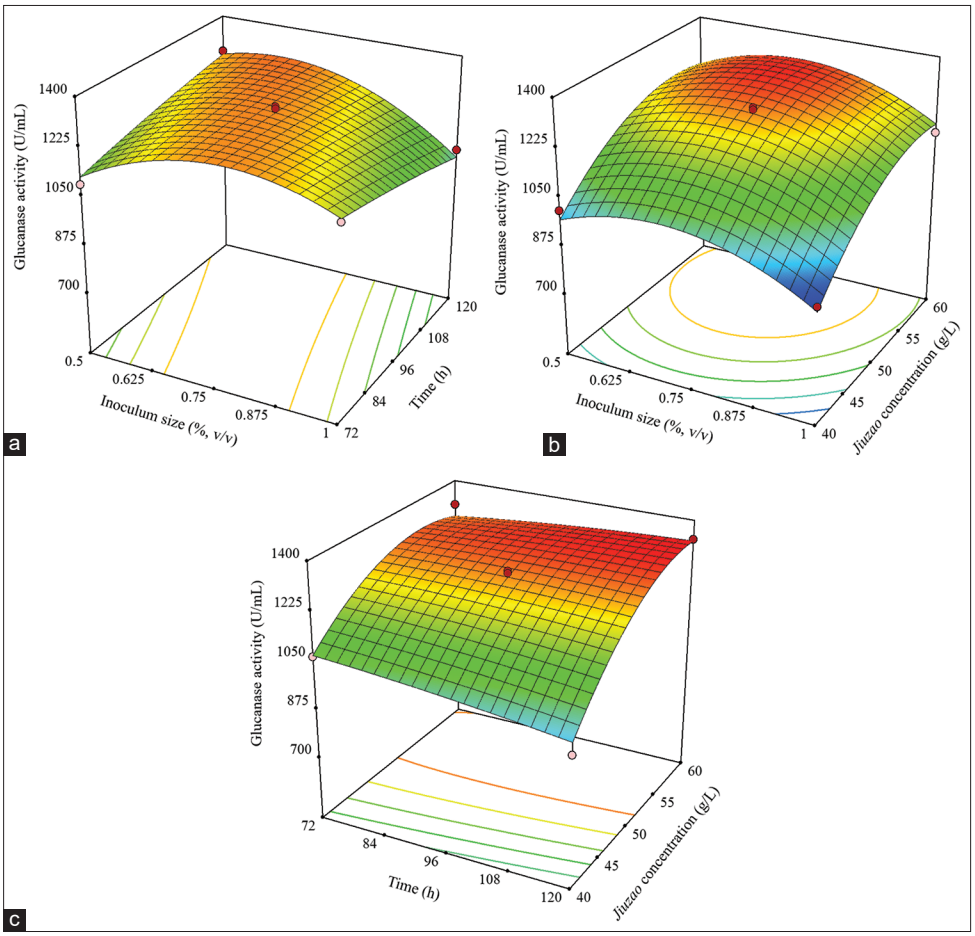


**Fig 11.** The response surface interaction of variable and its contour plots on the glucanase activity response using the Box-Behnken design. (a), interaction between inoculum size and time; (b), interaction between inoculum size and *Jiuzao* concentration; (c), interaction between *Jiuzao* concentration and time

$$Y=1269.67-41.63A-6.25B+132.38C-72.00AB+8.25AC+37.00BC-114.46A^2-8.21B^2-98.96C^2 \quad (2)$$

where Y was the glucanase activity.

The ANOVA showed that the regression model was highly significant ($P$ value was 0.0046) and the lack of fit was not significant with a $P$ value of 0.2593, indicating that the model was adequate for prediction within the range

of variables used. The determination coefficient ($R^2$) was 0.9625 indicating that the result variation of 96.25% for glucanase production was attributed to independent factors and only 3.75% of the total variation couldn't be explained by the model. In other words, a close agreement between the experimental results and the theoretical values predicted by the equation. The ANOVA revealed that inoculum size and *Jiuzao* concentration had strong linear effect on the glucanase production ($P<0.05$). Inoculum size had a negative effect, while *Jiuzao* concentration had positive effects, which was consistent with results of PB design. The order of influence of three factors on glucanase activity was different with results of PB design as follows: *Jiuzao* concentration > inoculum size > time, which may be the difference of interaction between factors caused by different number of factors in two designs. The interaction between inoculum size and *Jiuzao* concentration had significant negative effect on glucanase activity, while no evidence of more interaction that would contribute to glucanase activity at a significant level. And, the quadric term of inoculum size and *Jiuzao* concentration had a significant negative effect. Response surface plots for glucanase production by the above model were shown in Fig. 11, which illustrated the interactions between two factors by keeping the third factor at zero level. All response surface graphs displayed that glucanase activity increased first then decreased as the value of each factor increased, indicating the maximum level for glucanase activity could be obtained. The optimal conditions for the highest of glucanase activity of 1353 U/mL were calculated from the 2nd-order polynomial equation by Design-expert 11 software with the following critical values: A (inoculum size)=0.63% (v/v), B (time)=120 h, and C (*Jiuzao* concentration)=58.4 g/L. Verification experiments were carried out at the optimal conditions and the glucanase activity was 1336 U/mL, which was closed to the predicted value. Optimization resulted in a 2.14-fold in glucanase activity compared to the highest level (624 U/mL) in single factor design.

## CONCLUSION

*B. pyrrocinia* is an excellent functional strain from the *Baijiu* brewing environment which has outstanding advantages in producing esters. To make better use of this strain in *Baijiu* brewing, other potential application functions need to be explored. Previous studies have reported that *B. pyrrocinia* possesses a good ability to produce glucanase. In the present study, the production of glucanase by *B. pyrrocinia* was investigated using *Jiuzao* as the carbon source which avoids the environmental pollution caused by handling *Jiuzao* waste improperly. Five factors, inoculum size, time, *Jiuzao* concentration, temperature and initial pH, were found to significantly affect the glucanase activity of *B. pyrrocinia* B1213 using single factor and PB designs. The optimal conditions for glucanase production from *B. pyrrocinia* B1213 were obtained by the steepest ascent path design and RSM. The highest level of glucanase activity was 1336 U/mL under the following optimal conditions: *Jiuzao* particle size, 40-60 mesh; *Jiuzao* concentration, 58.4 g/L; urea concentration, 8 g/L; initial pH, 6.0; loading volume, 15 mL/250 mL; inoculum size, 0.63%; and temperature, 26 °C with shaking at 160 rpm for 120 h. This level of glucanase activity was 114% higher than that obtained under the cultivation conditions in a single factor design. This study has established a foundation for understanding the further degradation of *Jiuzao* by glucanase produced by *B. pyrrocinia* B1213 to produce valuable products, thereby helping to make full use of *Jiuzao*. And, the glucanase produced by *B. pyrrocinia* B1213 would be used to improve the efficiency and yield of *Baijiu* by exploring the degradation effect of cellulose in *Baijiu* production materials in future.

### Authorship contribution statement

Cheng Ruiwen, Wang Fuqiang, Xu Yiren and Wei Lai carried out the experiments. Yang Ran conceived of the study, designed the experimental protocol, and wrote the manuscript. Ma Jinghao, and Gao Peng revised the manuscript. Fan Guangsen and Liu Xiaoyan analyzed the data.

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### Data availability

All data and materials have been provided in this manuscript.

## COMPLIANCE WITH ETHICAL STANDARDS

### Conflict of interest

The authors report no declarations of interest.

### Ethical approval

Not applicable.

### Consent to participate

Not applicable.

### Consent for publication

The final manuscript is read and approved to submit to this journal by all authors.

### Code availability

Not applicable.

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## SUPPLEMENTARY TABLE

**Supplementary Table S1: Factors and levels of single factor design**

| Factor | Level/type |
|---|---|
| *Jiuzao* particle size (mesh) | >10, 10-20, 20-40, 40-60, 60-80, 80-100 and <100 |
| *Jiuzao* concentration (g/L) | 20, 30, 40, 50, 60 and 70 |
| Nitrogen source type | Yeast extract powder, tryptone, $NaNO_3$, $NH_4Cl$, beef peptone and urea |
| Urea concentration (g/L) | 2, 4, 8, 16 and 32 |
| Initial pH | 3, 4, 5, 6, 7, 8, 9 and 10 |
| Inoculum size (%, v/v) | 0.2, 0.5, 1.0, 2.0, 4.0 and 8.0 |
| Loading volume (mL/250 mL) | 15, 30, 45, 60, 75 and 90 |
| Shaking speed (rpm) | 120, 140, 160, 180, 200 and 220 |
| Temperature (℃) | 20, 25, 30, 35 and 40 |
| Surfactant type | Tween 20, Tween 40, Tween 60, Tween 80, Triton 100, Triton 114, glycerol and control |
| Time (h) | 24, 48, 72, 96, 120 and 144 |