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Fermentation optimization of rennet-producing *Bacillus amyloliquefaciens* GSBa-1 for high-density culture and its kinetic model

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Abstract

The milk-clotting enzyme (MCE) produced by *Bacillus amyloliquefaciens* GSBa-1 achieved the scale-up criterion from 0.25-L erlenmeyer flask to 5-L bioreactor sucessfully and exhibited remarkable milk-clotting activity (MCA) (626.3 SU/mL) that was 1.45-fold higher than the control by optimizing both medium composition and fermentation conditions.The growth of GSBa-1 was optimized through response surface methodology employing Plackett-Burman design and Box-Behnken design. The final optimized fermentation conditions were as follows: maltose (7.258g/L), corn steep liquor (8.15g/L), and Tween 80 (1.97) g/L; constant fermentation pH (7.0), temperature (45 °C) and stirrer speed (200 rpm). Kinetic models of microbial growth (X), product formation (P), and substrate consumption (S) were constructed, respectively. The derived model was the first reported model for GSBa-1 rennet production, contributing visual description for the rennet fermentation process, as well as the improvement of its productivity and efficiency.

Keywords: *Bacillus amyloliquefaciens* GSBa-1; rennet; fermentation optimization; high-density culture; kinetic model.

Practical Application: The rennet produced by *B. amyloliquefaciens* GSBa-1 is a promising alternative to calf rennet in processing of cheese.

1 Introduction

Bacillus amyloliquefaciens belongs to the super kingdom of bacteria with a *Bacillaceae* family and genus of *Bacillus* (Teng et al., 2017; WoldemariamYohannes et al., 2020). Even though *B. amyloliquefaciens* has different strains with different properties and applications, it is considered as a safe and nontoxic producing microbe (Chen et al., 2020; Shahzad et al., 2020; Tran et al., 2020). The rennet produced by *B. amyloliquefacies* GSBa-1 is a promising alternative to calf rennet in cheese processing (Teng et al., 2019; Zhao et al., 2020a). In order to apply this rennet in cheese production properly, it is necessary to optimize the conditions for the high milk-clotting activity rennet produced by *B. amyloliquefaciens* GSBa-1(Mota et al., 2018). High-density fermentation technology has been widely used in the fermentation optimization of eukaryotic microorganisms, prokaryotic microorganisms and algae, which can significantly increase the yield of microorganisms (Freudenberg et al., 2021; Kleman & Strohl, 1994; Liu et al., 2019; Wang et al., 2020). And the fermentation of microorganism is highly dependent on the composition of medium as well as their fermentation conditions (Ju et al., 2018; Martinez-Burgos et al., 2021; Wei et al., 2009).

Medium optimization is a very practical and valuable tool for fermentation industry to improve product yield and minimize by-products as well as reduce overall manufacturing costs. The metabolites produced by microorganisms mainly depend on the strain, the composition of the culture medium and culture conditions. Medium composition has a significant impact on the formation of target products (Jung & Lee, 2020; Rajeswari et al., 2015). Statistical optimization is a good mean screening significant variables in a large experimental design (Abdel-Fattah et al., 2005; Saavedra et al., 2021). Variables with statistically significant effects on milk-clotting activity were identified by screening using the Plackett-Burman design (PBD) (Elsayed & Abdelwahed, 2020; Jiang et al., 2020; Plackett & Burman, 1946). These were further optimized by Box-Behnken Design (BBD) experiments (Yang et al., 2020) and Response Surface Methodology (RSM) (Ibrahim et al., 2019; Zhao et al., 2020b).

Kinetic modeling has long been used to provide crucial information about the metabolic capabilities of microorganisms during their cultivation (Olivares-Marin et al., 2018; Zhang et al., 2019). In order to further study the internal laws and dynamic changes during the fermentation of *B. amyloliquefaciens* GSBa-1, mathematical models were used to quantitatively analyze the changes in bacterial growth, milk-clotting activity and the consumption of total sugars during the fermentation process.

In our previous research, the rennet from *B. amyloliquefaciens* GSBa-1 has been proved to have better metal ion chelating ability and antioxidant activity compared with commercial rennet. It improved biological activity of cheese while meeting general cheese requirements (Zhao et al., 2020a). In addition, GSBa-1 rennet cheese has slightly stronger proteolytic activity, which leads to more types flavors than those made with commercial rennet (Teng et al., 2019). Therefore, GSBa-1 rennet has great

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application potential in cheese production. In this study, the high-density culture strategy was approached by optimizing the composition of the medium and fermentation conditions, taking the milk-clotting activity as an indicator. At the same time, monitoring and understanding the fermentation characteristics of *B. amyloliquefaciens* GSBa-1 and establishing its fermentation kinetic model laid the foundation for the application of *B. amyloliquefaciens* GSBa-1 rennet in food industry.

2 Materials and method

2.1 Microorganisms and medium

B. amyloliquefaciens GSBa-1 was isolated from wine koji. The strain was stored in 50% glycerol at -80 °C.

The seed culture medium contained the following per liter: tryptone (10 g/L), yeast extract (5 g/L), and sodium chloride (10 g/L). The pH was adjusted to 7.0 and then autoclaved at 121 °C for 20 min.

The fermentation medium contained maltose (6.74 g/L), beef extract (8 g/L), corn syrup (7.5 g/L), Tween 80 (1.9 g/L). The pH was adjusted to 7.0 and then autoclaved at 121 °C for 20 min.

2.2 Erlenmeyer flask culture research

In order to develop a medium suitable for the production of high-activity rennet from *B. amyloliquefaciens* GSBa-1, the medium(carbon source, nitrogen source and inducer) was optimized in an Erlenmeyer flask.

2.3 Identification of the most significant nutrients by PBD

The PBD plays an important role in the rapid determination of key components in the medium (Singh et al., 2009). The four main variables of the current research, namely corn steep liquor, maltose, beef extract, and Tween 80, were represented by X_1, X_2, X_3, and X_4, respectively (Table S1) . Variables were evaluated at high (+1) and low (-1) levels. According to the model, 12 experimental runs were carried out. The PBD was based on the first-order polynomial equation (Equation 1):

$$Y = \beta_0 + \Sigma \beta_i X_i \quad (1)$$

where Y is the activity of GSBa-1 rennet, β_0 is the coefficient of the model, β_i is the linear coefficient, and X_i is the level of each independent factor.

From the regression analysis, variables that were significant at or above 95% level and at probability value of $p < 0.05$ were considered to have a great impact on the milk-clotting activity of GSBa-1 and were further statistically optimized by BBD.

2.4 Box-Behnken Design (BBD)

BBD is one of the RSM design with a three-level factorial design, which has been used as the experimental design model to optimize the influential parameters for improving milk-clotting activity (Wang et al., 2019). According to the results of PBD experiment, the levels of the variables and the experimental design were shown in Table S2. BBD was used to investigate the combined effect of three variables such as maltose, corn syrup, and Tween 80 concentration on rennet production. The corresponding fermentation was performed in 500 mL shake flasks with 50 mL medium and incubated at 30 °C in a shaker (160 rpm) for about 24 h. The broth was refrigerated at 4 °C and centrifuged at 10,000 rpm for 10 min, and the supernatant was obtained as crude enzyme solution that was used for milk-clotting activity analysis.

The accuracy of the fitted model was proved by analysis of variance (ANOVA) and the coefficient of R^2. The meaning of all terms in the polynomial model is calculated by calculating the statistical judgment F in the probability-value (value) is 0.05. Minitab software 17.0 is used to regress experimental data and generate 3D contour plots. These plots are generated by keeping two variable constants at 0 level and changing other variables within the scope of the experiment. The experiment was performed in triplicate, and the average response was used for analysis. Minitab was used for experimental design and analysis. Design-Expert 11.0 was used to design the experiment and analysis data.

2.5 Fermentation parameters optimization in bioreactor

The culture was used to inoculate the pre-prepared seed culture (3%, v/v) to optimize fermentation of natural pH medium cultivating in the fermenter (BioFlo 115, 5 L vessel volume, China) containing 3 L of minimal medium. After 24 h of fermentation, the broth was refrigerated at 4 °C and centrifuged at 10,000 rpm for 10 min, and the supernatant was obtained as crude enzyme solution that was used for milk-clotting activity analysis. The effects of fermentation conditions (pH, temperature and stirrer speed on milk-clotting activity of *B. amyloliquefaciens* GSBa-1 were studied to further improve the milk-clotting activity in the bioreactor.

2.6 Kinetic model of B. amyloliquefaciens GSBa-1

At present, various structured and unstructured kinetic models have been applied in microbial fermentation (Phukoetphim et al., 2017; Procentese et al., 2015; Zhou et al., 2020). The kinetic modeling of the fermentation process aims to describe the time evolution of different metabolite concentrations by explaining the formation and consumption rates of different metabolites in the metabolic pathway (Buehler & Mesbah, 2016; Thilakavathi et al., 2007). Unstructured models make it easier to monitor and predict batch fermentation processes than structured kinetic models (Phukoetphim et al., 2017).

Logistic equation is usually used to express the growth kinetics of microorganisms, which describes the typical S-shaped curve of strains. The Logistic model is not affected by the substrate, and can better show the influence of the change of the bacterial content on its growth during the fermentation process (Tashiro & Yoshimura, 2019). The GSBa-1 has an S-type growth curve, so the Logistic equation is used to express the growth law of *B. amyloliquefaciens* GSBa-1. The logistic equation is (Equation 2):

$$\frac{dX}{dt} = \mu m\left(1 - \frac{X}{X_m}\right)X \quad (2)$$

where dX/dt is cell growth rate (cfu·mL^{-1}·h^{-1}), t is the fermentation time (h), X is the cell concentration (× 10^7 cfu·mL^{-1}), X_m is the maximum cell concentration (× 10^7 cfu·mL^{-1}), μ is the specific growth rate (h^{-1}), μ_m is the maximum specific growth rate (h^{-1}). When t = 0, X_0 is the initial biomass concentration (× 10^7 cfu·mL^{-1}), the integrated form of Equation 2 can use $X = X_0$ gives a sigmoidal variation of X as a function of t (Equation 3), which can represent both exponential- and stationary-phase.

$$X = \frac{X_0 e^{bt}}{1-(1-e^{bt})\frac{X_0}{X_m}} \tag{3}$$

The Luedeking-Piret equation is widely used to characterize the relationship between product formation and microbial growth (Dhagat & Jujjavarapu, 2015). GSBa-1 rennet also uses the Luedeking-Piret equation to describe its synthesis kinetics. The Luedeking-Piret equation is (Equation 4):

$$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X \tag{4}$$

where P is rennet activity (SU·mL-1), α and β are rennet synthesis kinetic parameters, α is the product synthesis coefficient coupled with bacterial growth rate, β is coupled with bacterial concentration Product synthesis constant. When t = 0, P_0 is the initial rennet activity (SU·mL^{-1}), the integrated form of Equation 3 can be changed to Equation 5.

$$P = P_0 - \alpha X + \alpha\frac{X_0 X_m e^{\mu_m t}}{X_m - X_0 + X_0 e^{\mu_m t}} + \frac{\mathrm{B}X_m}{\grave{\imath}_m}\ln\left(\frac{X_m - X_0 + X_0 e^{\mu_m t}}{X_m}\right) \tag{5}$$

B. amyloliquefaciens GSBa-1 consumes the substrate, which is used for bacterial growth, maintenance of bacterial life activities and rennet formation. The Luedeking-Piret modified equation was used to describe the substrate consumption process of the rennet produced by GSBa-1. The Luedeking-Piret modified equation is (Equation 6):

$$-\frac{dS}{dt} = \frac{\mathrm{dX}}{\omega \mathrm{dt}} + \gamma X \tag{6}$$

where S is the substrate concentration (g·L^{-1}), ω is the yield constant of the substrate for bacterial growth, γ is the yield constant of the formation of rennet.

When t = 0, S0 is the initial substrate concentration (g/L), the integrated form of Equation 6 can be changed to Equation 7.

$$S = S_0 + \frac{X_0}{\grave{\mathrm{u}}} - \frac{X_0 X_m e^{\mu_m t}}{\grave{\mathrm{u}}\ (\mathrm{X}_m - X_0 + X_0 e^{\mu_m t})} + \frac{\gamma X_m}{\mu_m}\ln\left(\frac{X_m - X_0 + X_0 e^{\mu_m t}}{X_m}\right) \tag{7}$$

These model parameters were estimated by Origin software (2018 version) and used for nonlinear fitting.

2.7 Milk-clotting activity assay

Milk-clotting activity in the fermentation broths was analyzed according to Arima's (Yu et al., 2014) method. Two milliliters of 0.01 mol/L $CaCl_2$ solution containing 10% skimmed milk was accurately measured and kept it at 35 °C for 5 min, and two hundred microliters of fermented crude enzyme solution was added and mixed quickly. The time between addition and appearance of clots was recorded. The formula for calculating milk-clotting activity is as follows (Equation 8):

$$\mathrm{MCA} = \frac{2400}{\mathrm{T}} \times \frac{5}{0.5} \times D \tag{8}$$

where MCA is the rennet activity, SU·mL^{-1}; T is the coagulation time, s; D is the dilution multiple of the crude enzyme solution.

3 Results and discussion

3.1 Evaluation of the most significant factors affecting rennet activity

The optimization of key nutrients in fermentation medium for rennet production by *B.amyloliquefaciens* GSBa-1 using PBD was carried out in submerged cultures. Plackett-Burman design was carried out using MINITAB 17.0 software (Minitab, LLC, State College PA, USA) . The number of tests selected was N = 12, with four parameters X_1, X_2, X_3, and X_4 representing the maltose, corn steep liquor, beef extract, and Tween 80, respectively (Table S1). It can be seen that the obtained milk-clotting activities of the rennet ranged from 375 SU/mL to 437.125 SU/mL, which reflected the importance of medium optimization to enhancing milk-clotting activity.

The results of statistical analysis on the main effect were given in Table S3. The significance of each factor was assessed using the P-value at the significance level of 0.05. Pareto chart displayed that the significance of the five variables in terms of their effects on the milk-clotting activity. It was ranked as: B (Maltose) > A (Corn steep liquor) > D (Tween 80) > C (Beef extract) (Figure 1). Bars extending beyond this line correspond to statistically significant effects at a confidence level of 95% (Mayerhoff et al., 2006). Accordingly, the three variables were further considered for final optimization using BBD.

3.2 RSM optimization for enhancing B. amyloliquefaciens GSBa-1 rennet activity

Based on the PBD and single factor test results, the BBD was used for optimization with maltose and corn syrup, maltose and Tween 80, corn syrup and Tween 80 as variables, and the following regression equation prediction model is obtained with quadratic multiple regression simulation after integration (Equation 9):

$$\begin{aligned} Y &= 602.33 + 45.72A + 26.16B + \\ &31.75C - 30.58AB - 24.87AC + \\ &10.19BC - 59.04A^2 - 40.6B^2 - 49.72C^2 \end{aligned} \tag{9}$$

where Y is milk-clotting activity (SU/mL), A is maltose concentration (g/L), B is corn steep liquor concentration (g/L), and C is Tween 80 concentration(g/L), respectively.

The results were analyzed by variance analysis, and statistical significance was tested by F value. It has been shown from the obtained result that the model was highly significant, as suggested by the calculated F value (7.08) and low probability value (< 0.0001). The lack of fit F value of 0.3112 indicated that

this value is not significant to the pure error. The coefficient of determination (R^2) was calculated as 0.9758 for enhancing milk-clotting activity, representing that the statistical model can explain the adequate variability in the response (Table S4).

Furthermore, three-dimensional response surface plots showed the interactive effects of the three significant fermentation factors on the milk-clotting activity (Figure 2). It indicated that the maximum milk-clotting activity should occur with medium levels of maltose, corn syrup and tween 80. We found that the interactions between maltose and corn syrup (AB), and maltose and tween 80 (AC) were elliptical, indicating that a substantial correlation between each two variables and their influence on improving milk-clotting activity. The result was same as the p-value in Table S4. But The corn syrup and tween 80 (BC) contour lines were round, indicating a lack of significance. With the help of numerical optimization, predicted maximum milk-clotting activity (616.657 SU/mL) could be obtained with 7.258 g/L maltose, 8.15 g/L corn syrup and 1.97 g/L Tween 80. The milk-clotting activity by GSBa-1 is higher when compared with the thermophilic fungus N31 rennet (60.5 U/mL) and the fungal Quambalaria cyanescens rennet (117 SU/mL), but lower than a novel BL312 milk-clotting enzyme (MCE) (865 ± 20 SU/mL) exhibited high-level expression by optimizing induction conditions in recombinant *Escherichia. coli* harboring pET24a-proMCE (Almeida et al., 2015; Wang et al., 2014; Zhang et al., 2021). What is more, bacterial MCEs have more potential than other MCEs due to their greater biochemical diversity and easier genetic modification. Therefore, the attempts should be made to increase the MCA/PA ratios of GSBa-1 MCEs by mutation or to study the changes in cleavage sites and functional peptides after genetic modification in the future.

3.3 Optimization of fermentation condition in bioreactor

Bioreactors are practical tools that are used for economical, time-conserving and large-scale production of biomass from cell cultivation (Manjarres-Pinzón et al., 2022). Different constant pH, stirrer speed and fermentation temperature on the activity of GSBa-1 rennet were investigated in a fermenter with 3 L effective volume (Figure 3).

The pH affects product growth and yield in microbial deep fermentation (Anggela et al., 2022; Kumar et al., 2010). Since the acidity of the fermentation broth was constantly changing during the fermentation process, the method of automatic feeding

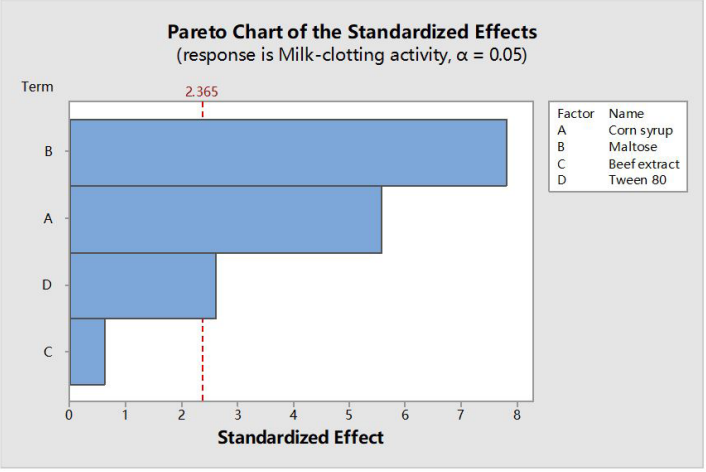

Figure 1. Pareto chart for the effect of medium nutrient components according to PBD.

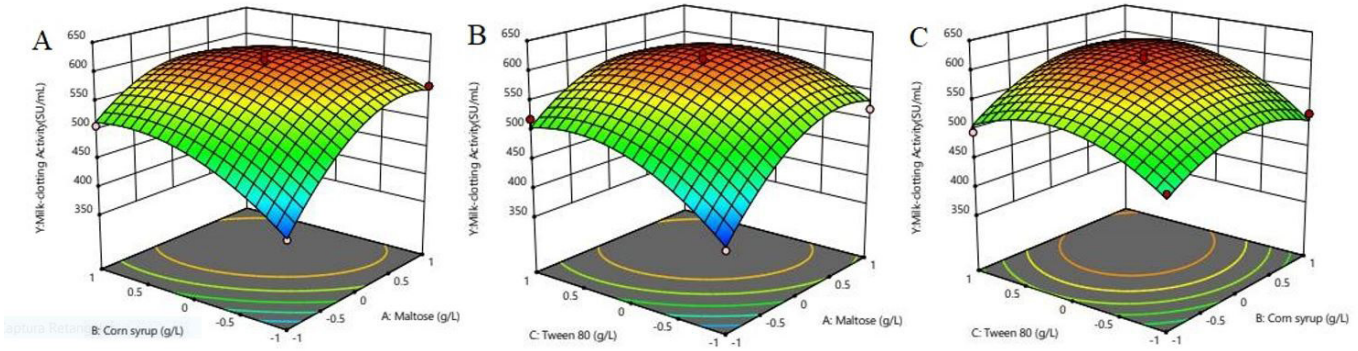

Figure 2. Three-dimensional response surface plots showing the influence of variable pairs on rennet activity: A-maltose and corn syrup, B-maltose and Tween 80, C-corn steep liquor and Tween 80.

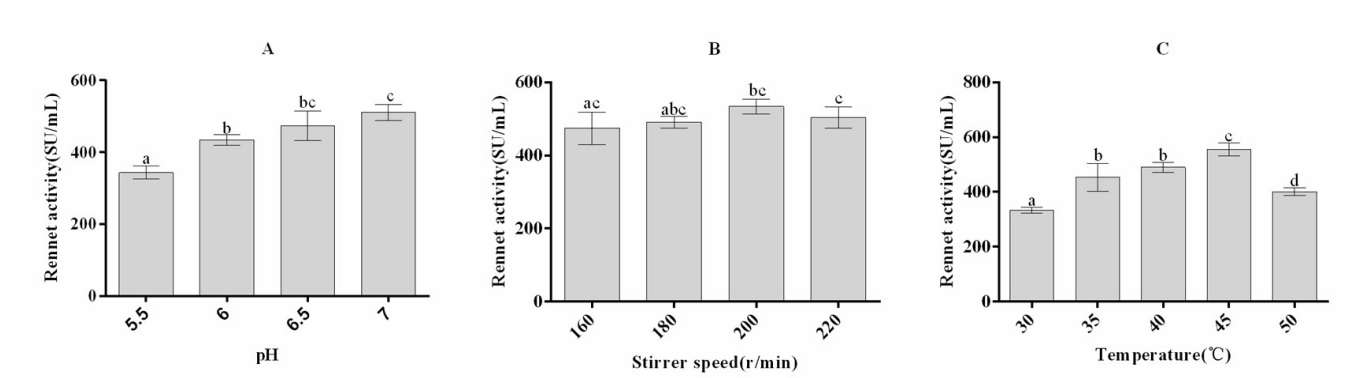

Figure 3. Optimization of fermentation parameters in bioreactor: A-pH, B-rotating speed, C-temperature. The bars represented by different letters are significantly different ($p < 0.05$).

was adopted in this experiment, and sodium hydroxide and hydrochloric acid were selected to control the pH to maintain a fixed value. Maximum milk-clotting activity of 525.3 SU/mL was obtained with the optimal pH around 7 (Figure 3A). In the acidic environment with a pH of 5.5, the milk-clotting activity of this broth decreased significantly, which indicated that the pH could significantly affect the rennet activity.

The shaker speed was related to the dissolved oxygen content in the culture solution (Ducros et al., 2009). Figure 3B showed that the maximum milk-clotting activity of *B. amyloliquefaciens* GSBa-1 was enhanced when a faster stirring rate (200 rpm) was applied as compared to the cell density under the stirring rate of 160 rpm. But by further increasing the stirring speed, the milk-clotting activity was decreased.

Milk-clotting activity was also significantly regulated by different temperature. Maximum milk-clotting activity of 540.98 SU/mL was obtained with the optimal temperature around 45 °C (Figure 3C), which is often the case that most strains share the same trend, despite that the optimal culture temperatures differ. Rennet bioprocess based on *B. amyloliquefaciens* GSBa-1 was successfully scaled up based on rennet activity as the scale-up criterion from 0.25-L erlenmeyer flask to 5-L bioreactor.

3.4 Fermentation characteristics of high-density culture of B. amyloliquefaciens GSBa-1 to produce rennet

The changes of OD value and MCA during the fermentation of GSBa-1 were observed (Figure 4). The growth rate of GSBa-1 increased rapidly after 2 h, and the logarithmic growth phase began; and after 12 h, it entered the stable phase. At this time, the OD value of the crude enzyme solution was 1.6024, and the milk-clotting activity was 501.33 SU/mL, which increased by 12% than before. At 24 h, the accumulation of metabolites of the strain reach the highest, and the activity of rennet reached the highest value, which was 626.3 SU/mL.

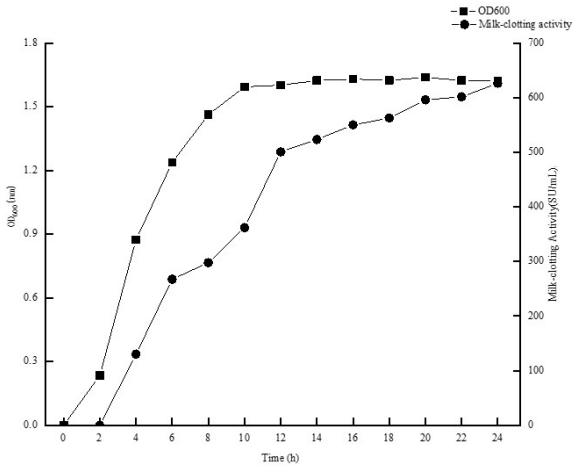

Figure 4. Changes of MCA and OD_{600} during the process of culturing GSBa-1 to produce rennet in a fermenter.

3.5 Fermentation kinetic parameter fitting

To be of value for industrial biotechnology, mathematical models should be able to assist in the rational design of cell factory properties or in the production processes in which they are utilized. Kinetic models are particularly suitable towards this end because they are capable of representing the complex biochemistry of cells in a more complete way compared to most other types of models. They can, at least in principle, be used to in detail understand, predict, and evaluate the effects of adding, removing, or modifying molecular components of a cell factory and for supporting the design of the bioreactor or fermentation process. The initial values of biomass concentration, milk-clotting activity and substrate concentration were derived from the experimental results with mean values of 130.54, 0 and 30.608, respectively. And other unknown parameters in Equations 4, 6 and 8 were estimated by fitting the experimental data obtained from the batch fermentation.

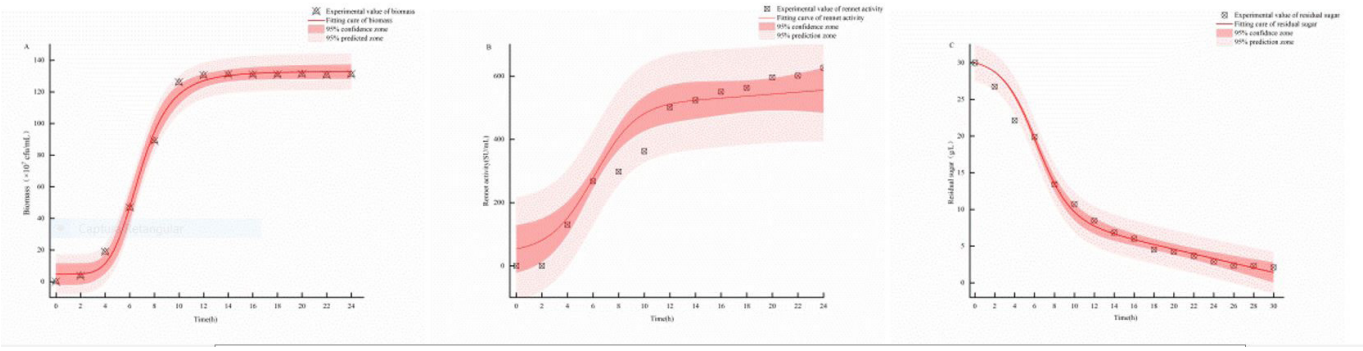

Figure 5. *B. amyloliquefaciens* GSBa-1 kinetic model fitting curve: A-GSBa-1 growth kinetic model, B-milk-clotting activity synthesis kinetic model, C-substrate consumption kinetic model.

Table S5 summarized the fitting curves of cell growth, milk-clotting activity and substrate consumption kinetics can be observed, and the R^2 values of the model equations were 0.9927, 0.9168 and 0.9846, respectively (Figure 5). The results showed that the test value and the predicted value of the model have a high degree of fit, and the selected model could well represent the changes of various substances during the batch fermentation of *B. amyloliquefaciens* GSBa-1.

4 Conclusion

Under the optimized medium and culture conditions, the GSBa-1 milk-clotting enzyme achieved the scale-up criterion from 0.25-L erlenmeyer flask to 5-L bioreactor successfully, and the milk-clotting activity of GSBa-1 rennet was increased to 626.3 SU/mL, which was an increase of 45.13% compared to that before optimization. What is more, a kinetic simulation model has been developed to predict accurately the dynamic behavior of metabolites in rennet fermentation by *B. amyloliquefaciens* GSBa-1 in the optimized medium. Therefore, these findings were valuable for the development of effective fermentation methods for the production of GSBa-1 rennet, and laid the foundation for the application of GSBa-1 rennet in the industrial production of cheese.

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Supplementary Material

Supplementary material accompanies this paper.

Table S1. PBD matrix of B. amyloliquefaciens GSBa-1 and the corresponding rennet activity.

Table S2. Coded levels and real values (in parentheses) for the BBD and lipase activity achieved after fermentation by B. amyloliquefaciens GSBa-1.

Table S3. Regression analysis of the Plackett Burman design for rennet production.

Table S4. Analysis of variance on regression model.

Table S5. Parameters of kinetic models for batch fermentation of B. amyloliquefaciens GSBa-1.

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