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Full Length Research Paper

Experimental analysis on the effect of addition of *Rhizoma gastrodiae* on mycelia and exopolysaccharide productions by submerged culture of *Grifola frondosa*

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The effect of addition of *Rhizoma gastrodiae* on mycelia and exopolysaccharide production by submerged culture of *Grifola frondosa* was investigated. About 7% (v/v) of *R. gastrodiae* extract was added to the basal medium used for its biomass and exopolysaccharide productions. The biomass and exopolysaccharide productions reached a maximum of 2.0630±0.0520 g/L and 89.3846±3.2422 mg/L, respectively after day 10 and 8 of cultivation. *G. frondosa* was sensitive to *R. gastrodiae*, and there was a reduction in the cycle of exopolysaccharide synthesis. The pH variation in culture provided an indirect inference to this stimulating effect of *R. gastrodiae* extract addition. Moreover, a curve fitting and parameter analysis was carried out to assay the increasing effect on biomass and exopolysaccharide productions. Meanwhile, the kinetics parameters predicted through the fitting equations in culture, X_0 = 0.1523 g/L, X_m = 2.0630 g/L, μ_m = 0.4994 d^{-1}, α = 0.0858 and β = -0.0069, further clarified the stimulatory effects of *R. gastrodiae* extract addition in *G. frondosa* culture.

Key words: *Rhizoma gastrodiae, Grifola frondosa*, biomass, exopolysaccharide.

INTRODUCTION

Grifola frondosa (*G. frondosa* or Maitake) is a Basidiomycete fungus belonging to the order *Aphyllophorales* and the family *Polyporeceae*. Its fruiting bodies, which are called "Huishu hua" in Chinese, "Maitake" in Japanese and "Hen of the Woods" in America, are rich in polysaccharide, and especially its exopolysaccharides (EPS) have attracted considerable attentions for their various physiological activities, such as anti-tumor, immunity, HIV infections, antioxidant and superoxide anion scavenging (Suzuki et al., 1989; Deng et al., 2009; Kodama et al., 2003; Nanba et al., 2000; Lin, 2011). The mycelia biomass (BIO) and EPS are two of the most desired products in submerged cultivation of *G. frondosa*, and it has become a hot topic to obtain EPS of *Grifola frondosa* in maximum amount in recent years (Hsieh et al., 2006, 2008). Meanwhile, to accelerate BIO and EPS productions, many studies in recent years have shown that proper amount of medicine added to the fungal culture can promote fungal growth or increase the production of active products (Li et al., 2006; Liu and Zhang, 2007; Kim et al., 2010).

Rhizoma gastrodiae (also called "Tianma" in Chinese) belongs to Orchidaceae, and it is one of the earliest and most important traditional herbal medicines in thousands of years. It has proved to have five major active components (Liu et al., 2002). Our research is supported by National Natural Science Foundation of China, and we have engaged in bio-transformation for many years, especially the bio-transformation between *G. frondosa* and *R. gastrodiae*, and have found that *R. gastrodiae* takes advantage of stimulating Maitake's biomass and

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EPS productions among various traditional Chinese medicines, such as *Thunb, Houttuynia*, *Buckwheat*, and so on. Consequently, *R. gastrodiae* was selected as the modeling medicine in this RESEARCH.

In this study, the mycelia growth and exopolysaccharide productions in *G. frondosa* liquid culture with *R. gastrodiae* extract addition were investigated. The fermentation kinetics of mycelia and EPS in culture with and without *R. gastrodiae* extracts were recorded and compared. Moreover, the experimental data of cell growth and products formation in culture with and without *R. gastrodiae* extract addition was predicted via the fitting equations in *G. frondosa* culture. A curve fitting and parameter analysis were carried out to identify the stimulatory effects of *R. gastrodiae* extract addition in *G. frondosa* culture.

MATERIALS AND METHODS

R. gastrodiae and its extracts

R. gastrodiae was purchased from a pharmacy in Guiyang, Guizhou province, China. It was washed, cooked and dried at 55°C before smashing and pounding to powder (40 mesh). For the preparation of extracts, 10 g of *R. gastrodiae* were extracted in 100 ml of 75% ethanol for 48 h. The extracts were filtered, and the ethanol was removed under reduced pressure to obtain dry extracts. Subsequently, 100 ml of water was used to re-dissolve the dry extracts. Then 10 ml of the extracts was obtained from 1 g of *R. gastrodiae*, before it was filtered and finally used for the submerged cultivation of *G. frondosa*.

Strain and culture conditions

G. frondosa strain (51616) was obtained from Microbial Culture Collection of China Agricultural Center (Beijing, People's Republic of China). It was grown in a 250 ml shake flask containing 100 ml seed cultures containing (g/L): glucose, 20; peptone, 2; KH_2PO_4, 2; $MgSO_4$, 1; and the initial pH was not adjusted. This was then used to incubate at 25°C for 9 days with shaking at 150 rpm. For the fermentation, the medium consisting of (g/L): glucose, 30; peptone, 5.5; KH_2PO_4, 1.5; $MgSO_4$, 0.75; an initial pH not adjusted and also containing *R. gastrodiae* extract (1 to 11% v/v), was sterilized and inoculated with the seed culture (15% v/v). The fermentation medium was incubated for 7 days.

Determination of biomass growth

Biomass was obtained by filtering the fermentation broth, washing the mycelia three times with distilled water, and drying at 60°C for a sufficient time to constant weight. The values reported were means of each determination.

Measurements of exopolysaccharide concentration

The EPS concentration was determined by adding 95% absolute alcohol into the filtrate and kept at 4°C for 24 h to precipitate the crude EPS. The mixture was separated by a centrifugation at 4,000 rpm for 15 min. The precipitate was rewashed with 80% (v/v) ethanol two times and dried to remove residual ethanol at 60°C.

Finally, the EPS content was determined by the phenol-sulphuric acid assay (Dubois et al., 1956).

Determination of pH value

The pH of the fermentation medium was determined using pH meter.

Drafting, modeling and statistical analysis

All the experimental data were graphed by Origin software (version OriginPro8.5.0 SR1; The OriginLab, EA). The fitted curves and model parameters were obtained by nonlinear fitting using the nlitool function from MATLAB software (version 7.10; The Math Works, Natick, MA). Reactions rates were solved iteratively at each time-step, updating the variables once convergence was achieved. To evaluate the fitted performance and accuracy of models, root mean squares error (RMSE) was employed (He et al., 2008):

$$RMSE = \sqrt{\frac{\sum\left(Y_{i,e} - Y_{i,p}\right)^2}{n}} \quad (1)$$

Where, $Y_{i,e}$ is the experimental data; $Y_{i,p}$ is the corresponding predictive data and n is the number of the experimental data. Here n = 3, and we aimed at minimizing RMSE.

RESULTS AND DISCUSSION

Effect of *R. gastrodiae* concentration on the cell growth and EPS production

To obtain the optimal conditions for *R. gastrodiae* extract addition, varied amounts of *R. gastrodiae* extracts were supplemented at the beginning of culture and the results obtained at day 7 are recorded in Figure 1. It was noted that both BIO and EPS productions increased with the increase of *R. gastrodiae* extract concentration from 0 to 7% (v/v), whereas further increase in extract concentration to 11% (v/v) exerted a negative effect on the production compared to that of 7% (v/v) *R. gastrodiae* extract addition. The maximum BIO and EPS productions obtained under 7% (v/v) *R. gastrodiae* extracts reached 1.2979±0.0440 g/L and 76.7762±2.1703 mg/L, and increased by 129.96 and 51.46% compared with the control containing the 0% (v/v) extracts, respectively. Hence, *R. gastrodiae* at 7% (v/v) markedly promoted the biomass and EPS biosynthesis of *G. frondosa*.
Accordingly, the level of 7% (v/v) *R. gastrodiae* extracts was selected for the subsequent studies.

Kinetics analysis of the *R. gastrodiae* extracts effect on mycelia and exopolysaccharide productions by *G. frondosa*

In order to explore the effects of *R. gastrodiae* extracts on

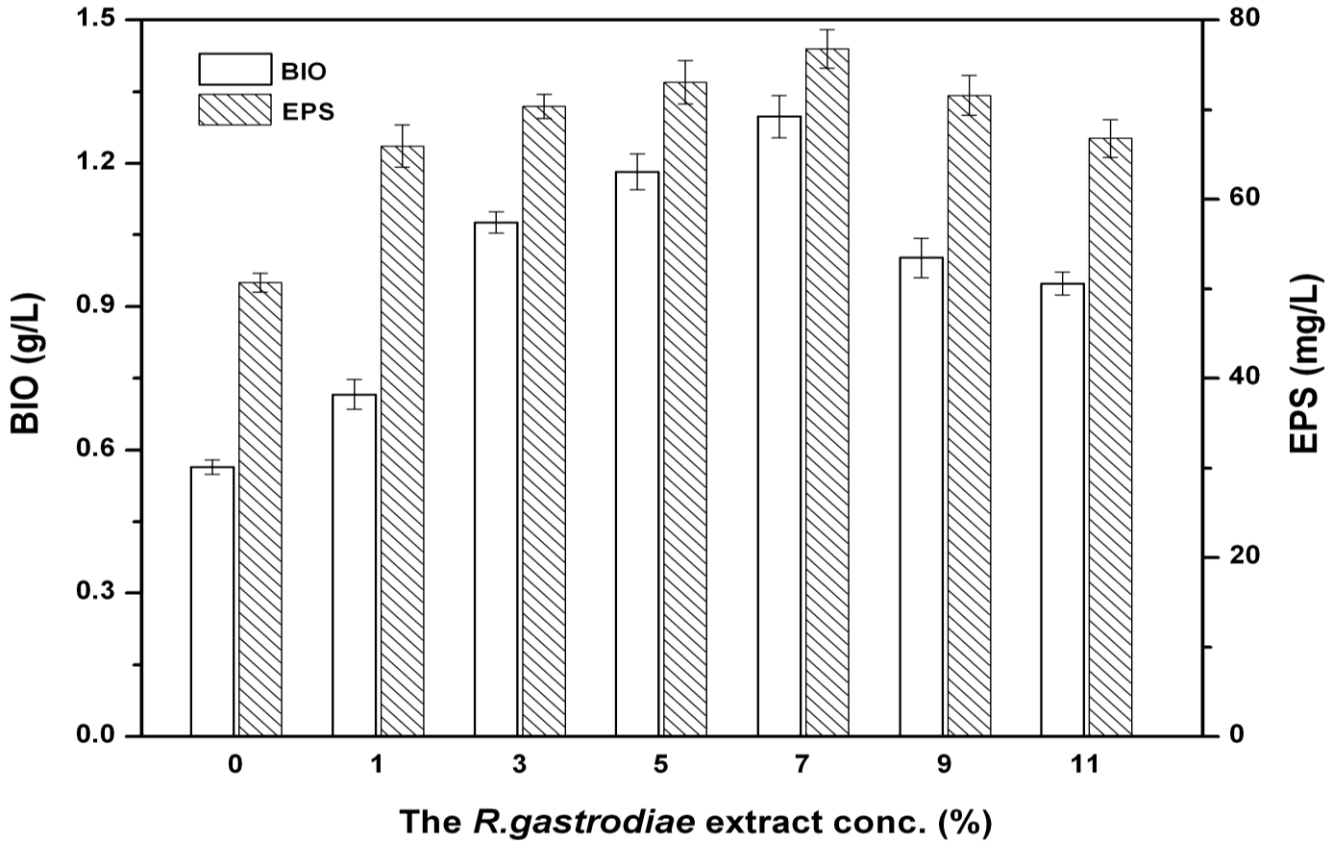

Figure 1. Effect of *R. gastrodiae* extract concentrations on biomass and EPS productions by *G. frondosa* culture. Cells were cultivated at 25°C for 7 days on a rotary shaker at 150 rpm. The error bar ranges denote the standard deviations of three trials.

G. frondosa submerged culture from a viewpoint of kinetics, liquid cultures with addition of 7% (v/v) *R. gastrodiae* extracts (the sample group) that were used as stimulus were compared to the cultures without *R. gastrodiae* extracts (the control group). Remarkably, as shown in Figure 2, *R. gastrodiae* extracts enhanced the growth and EPS production of *G. frondosa* simultaneously, and through the whole fermentation process. The highest amount of mycelia and EPS were increased to 2.0630±0.0520 g/L and 89.3846±3.2422 mg/L by adding 7% (v/v) *R. gastrodiae* extracts, accordingly increased by 169.32% and 52.49% compared with the control.

However, the respective top of BIO and EPS in the sample appeared on the 10th and 8th day, whereas in the control, BIO and EPS simultaneously reached their tops on the 10th day. So the synthesis of EPS seemed to be more synchronize with the cell growth on the whole in the control, perhaps the extract stimulated an earlier EPS production generating a curve displacement to the left in Figure 2. Furthermore, this also indicated that *R. gastrodiae* might reduce the cycle of Maitake's EPS synthesis, and EPS would not accumulate until the cell density met a certain level. So we can adjust the fermentation period according to our different purposes. Additionally, the sample's cell-growth and EPS curves seemed to be much steeper, also implying Maitake's sensitivity to *R. gastrodiae* due to a higher biomass and EPS production rate.

pH Profiles

The pH value is always considered as one of the critical indicators for mycelia growth and polysaccharide production during fermentation process. The pH profiles of both cultures (medium with and without *R. gastrodiae* extract additions) are shown in Figure 3. It was observed that the pH exhibited a sharp increase to the same value of 6.8 in both cultivations. However, the control remained at this high value from day 5 to 12; in contrast, the pH value of 6.8 in the sample continued only in 4 ~ 7 days, when BIO and EPS also happened to a rapid increase in both cultures. It was suggested that the pH value of 6.8 probably enhanced the growth of Maitake. However, further researches are being explored why there was a

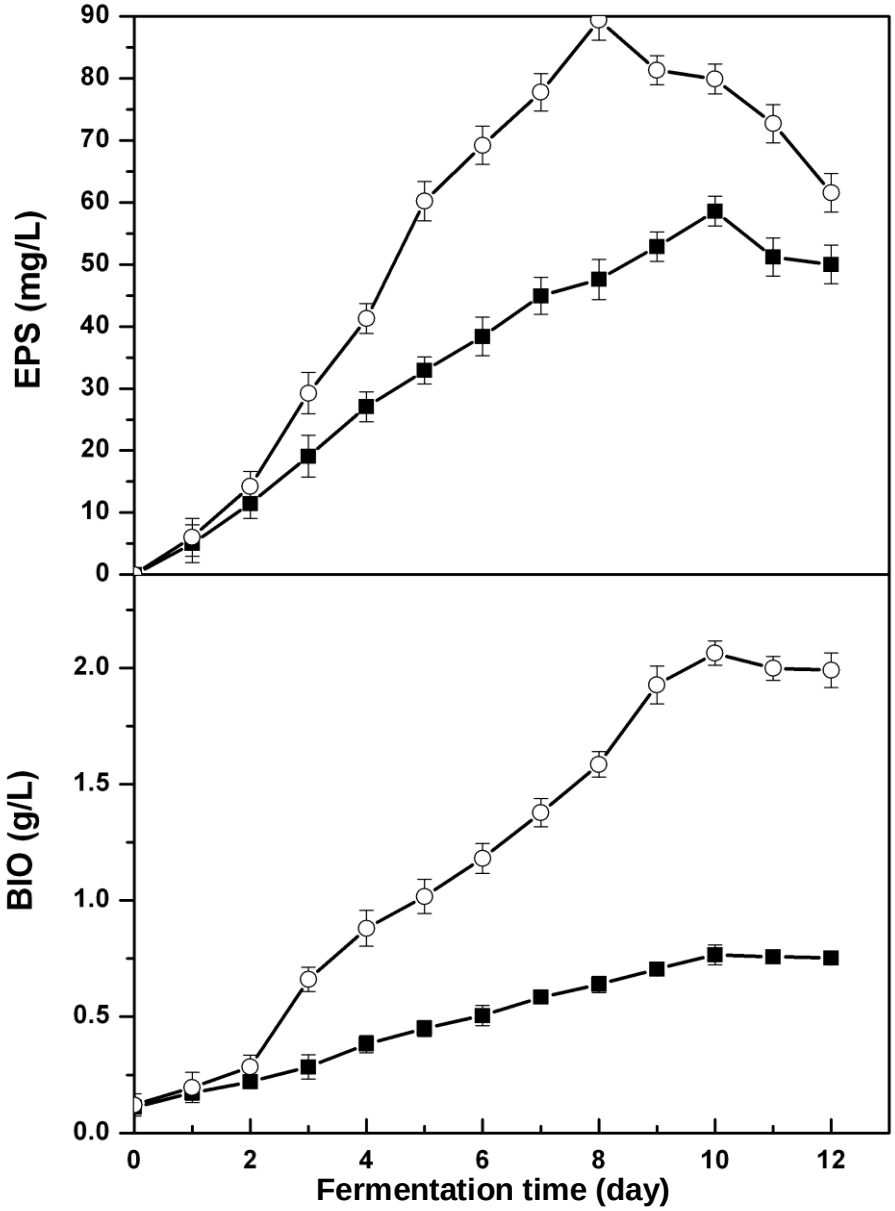

Figure 2. BIO and EPS of the control (■) and the sample (⊖) containing 7% (v/v) *R. gastrodiae* extract during the fermentation time. Cells were cultivated at 25°C for 12 days on a rotary shaker at 150 rpm. The error bar ranges denote the standard deviations of three trials.

slight decline at day 8. Meanwhile, a great fluctuation followed at the end of the experimental days, indirectly enlightening us of a stimulating effect of *R. gastrodiae* on *G. frondosa*.

Curve fitting and parameter analysis

Although a remarkable improvement on BIO and EPS productions was observed by *R. gastrodiae* extract addition, no research provided the numerical analysis on this promotion. Moreover, some fermentation parameters themselves could demonstrate the characteristics of both. In order to further establish the relationship of BIO and EPS productions coinciding with *R. gastrodiae* addition, the respective BIO and EPS produced from the control and sample groups were fitted based on Logistic and Luedeking–Piret equations (John, 1986; Robert and Edgar, 1959), and the regression equations are listed as follows:

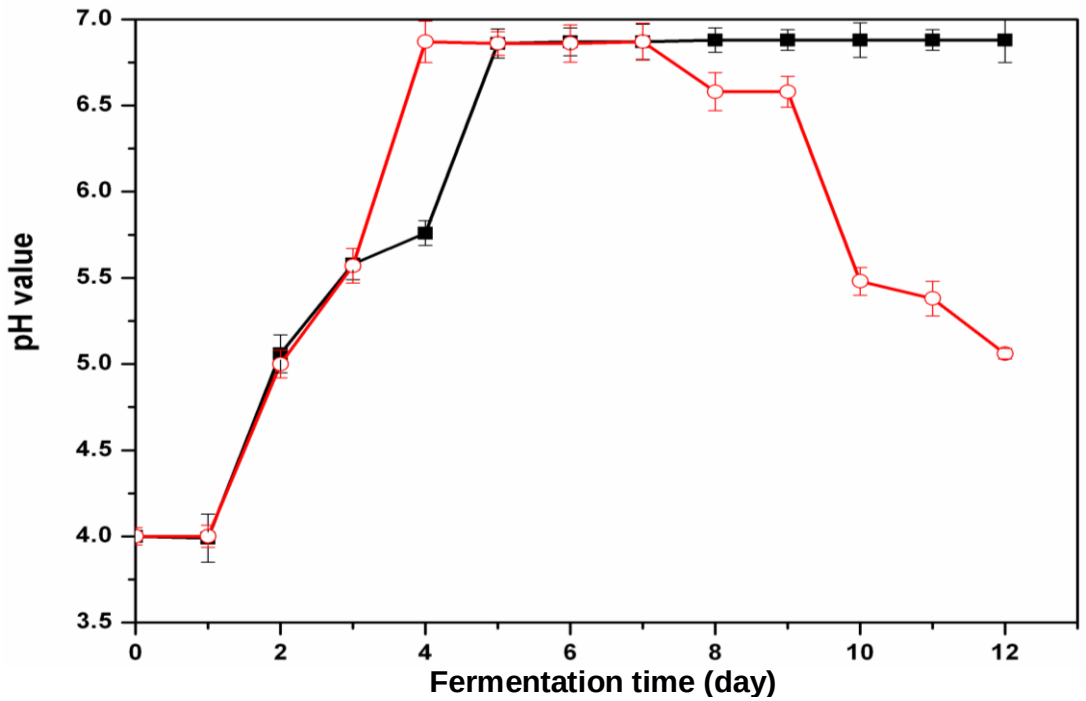

Figure 3. PH values of the control (■) and the sample (⊖) during *G. frondosa* culture. Cells were cultivated at 25°C for 12 days on a rotary shaker at 150 rpm. The error bar ranges denote the standard deviations of three trials.

$$CBIO_{(t)} = \frac{0.1065 \times \exp(0.4430 \times t)}{0.8610 \times [1 - \exp(0.4430 \times t)]} \quad (2)$$

$$CEPS_{(t)} = 105.9 \times CBIO_{(t)} - 4.7 \times \ln[0.8610 + 0.1390 \times \exp(0.4430 \times t)] - 11.3 \quad (3)$$

$$SBIO_{(t)} = \frac{0.1523 \times \exp(0.4994 \times t)}{0.9262 \times [1 - \exp(0.4994 \times t)]} \quad (4)$$

$$SEPS_{(t)} = 85.8 \times SBIO_{(t)} - 28.5 \times \ln[0.9262 + 0.0738 \times \exp(0.4994 \times t)] - 13.1 \quad (5)$$

Where, t is the fermentation time expressed in day; $CBIO_{(t)}$ and $SBIO_{(t)}$ are the biomass obtained in the control and sample cultures following with the fermentation time, respectively expressed in g/L; $CEPS_{(t)}$ and $SEPS_{(t)}$ are the exopolysaccharides produced in the control and sample cultures following with the fermentation time, respectively and expressed in mg/L. All the experimental data and the data predicted from Equations 2 to 5 are shown in Figure 4. The strikingly close estimation of Equations 4 and 5 to the experimental data in Figure 4C and D implies that the promotion of BIO and EPS productions with *R. gastrodiae* extract addition can be directly regarded as a linear combination of their individual cultures. Moreover, only the fitting curve of S-EPS appeared the top among four figures, and this implied the synthesis of EPS was more synchronize with the cell growth in the control.

On the other hand, as observed from Table 1, the parameters of X_m, X_0 and μ_m from the sample are larger than the control's. This might infer that *R. gastrodiae* extracts significantly contributes to BIO and EPS productions. However, a negative β also implied a mutual inhibition between growth and product synthesis, which was also proved in Figure 2. Moreover, α closer to the zero value and β farther to the zero value in the sample re-suggested that the control will follow a combined growth-associated behavior, and the growth and product synthesis inter-depend more complexly and sensitively. Accordingly, these numerical results further clarify the *R. gastrodiae* stimulatory effects in *G. frondosa* culture.

Conclusion

To date, many reports have focused on the addition of Chinese medicine to stimulate mycelia growth and polysaccharide productions for several medicinal fungi cultivations. *R. gastrodiae* as one of the latest stimulus is being well-known. However, all the outcomes were based on observation. In this study, an experimental analysis

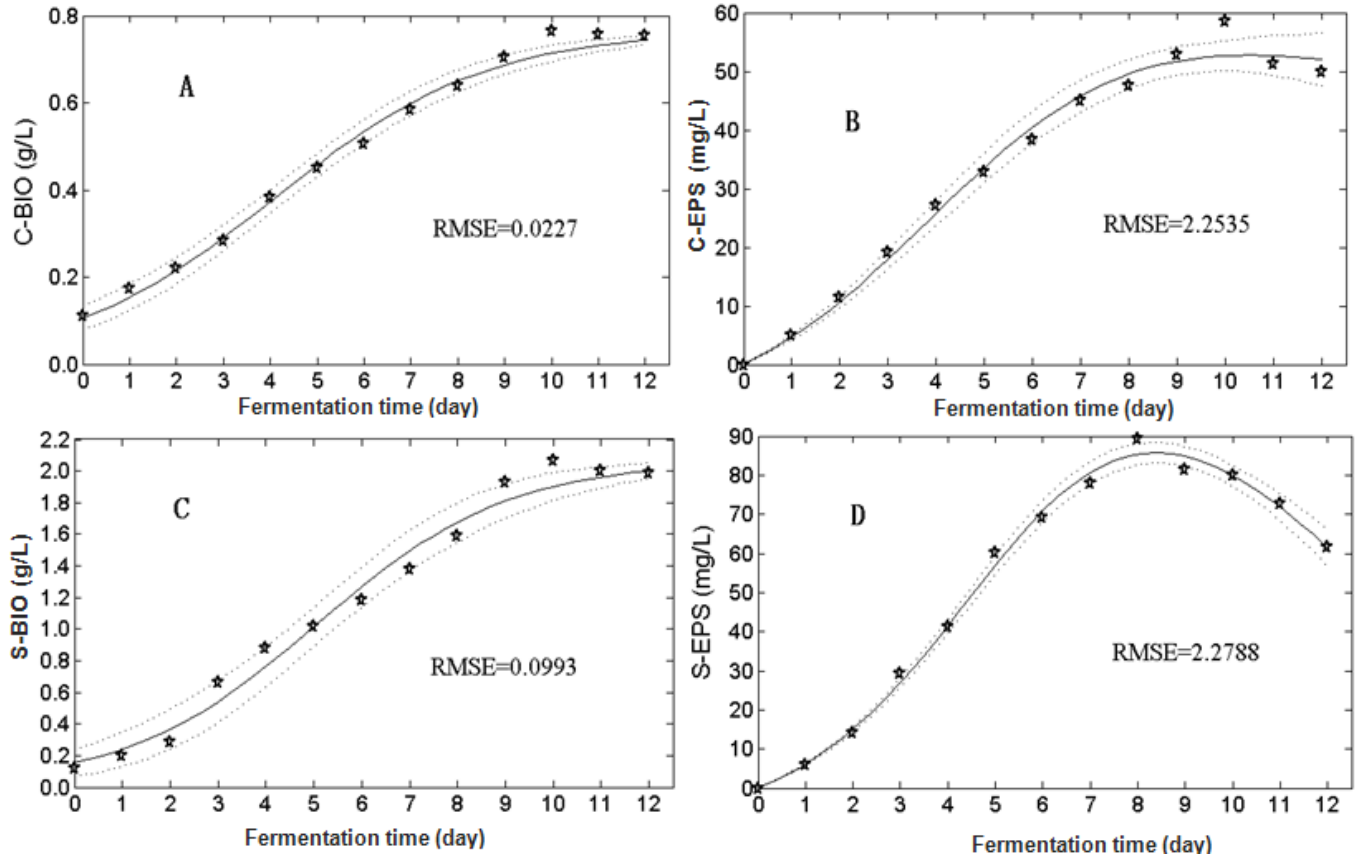

Figure 4. Comparison of experimental data (★) with the prediction lines (—) calculated via simulation equations, separately from the control (C-BIO and C-EPS) and sample (S-BIO and S-EPS). Here, the final fitting curves were drawn by three comprehensive considerations: the authenticity of experimental data, the distribution of initial values at confidence interval for 95% (------) and the overall minimum RMSE for each simulated component.

Table 1. Comparison of kinetics parameters estimated from the control and sample groups[a].

Parameter[b]	C-Value	S-Value	Note
X_0	0.1065	0.1523	The sample group increased by 43.00% than the control.
X_m	0.7660	2.0630	The sample group increased by 169.32% than the control.
μ_m	0.4430	0.4994	The sample group increased by 12.73% than the control.
α	0.1059	0.0858	The sample group was reduced than the control, yet was closer to the zero value.
β	-0.0027	-0.0069	The sample group was reduced than the control, yet was farther from the zero value.

The C-Value is the fitted value of the control; S-Value is the fitted value of the sample. [a]The culture harvested in the sample groups used additional 7% (v/v) *R. gastrodiae* extracts as stimulus, and the control without *R. gastrodiae* extracts in the culture; [b] X_0 is the initial cell population size, g/L; X_m is the maximum attainable biomass concentration, g/L; μ_m is the maximum specific growth rate, d^{-1}; α and β are the product formation constants. The larger is the α value, the more growth-associated is the fermentation process, while the β value represents the non-growth-associated process, and they are both model parameters, changing with the different fermentation conditions. Here, the final parameters were estimated using a best-fit approach with initial values placed at 95% global confidence bands, and iteratively adjusted to obtain the most accurate correlations.

about the promotion by *R. gastrodiae* extract addition was proposed. The results obtained showed that 7% (v/v) *R. gastrodiae* extract addition can promote the mycelia growth and EPS production of *G. frondosa* most. Furthermore, a kinetics analysis implied that *R. gastrodiae* extract addition might reduce the cycle of Maitake's EPS synthesis. From the fitting curves and kinetics parameter analysis, the promotion of BIO and EPS productions with *R. gastrodiae* extract addition can be referred to directly as a linear combination of their individual cultures. Hence

from the practical viewpoint, the addition of *R. gastrodiae* not only enhances *G. frondosa* BIO and EPS productions but can also help to adjust the fermentation period according to different purposes and further shortens the cultivation time. These results would give benefits to the *G. frondosa* culture for further applications in both academy and industry.

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