Optimization of Soluble Organic Selenium Accumulation during Fermentation of *Flammulina velutipes* Mycelia

Yunfeng Ma[a,§], Fu Xiang[b,§], Jun Xiang[b], and Longjiang Yu[c,*]

[a] College of Life Science, Henan University, Kaifeng, 475004, China
[b] Key Laboratory of Economic Forest Germplasm Improvement and Resources Comprehensive Utilization, College of Chemistry and Life Science, Huanggang Normal University, Huanggang, 438000, China
[c] College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, 430074, China. E-mail: xiangfu@hgnu.edu.cn

* Author for correspondence and reprint requests

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Selenium is an essential nutrient with diverse physiological functions, and soluble organic selenium (SOS) sources have a higher bioavailability than inorganic selenium sources. Based on the response surface methodology and central composite design, this study presents the optimal medium components for SOS accumulation in batch cultures of *Flammulina velutipes*, *i.e.* 30 g/L glucose, 11.2 mg/L sodium selenite, and 1.85 g/L NH_4NO_3. Furthermore, logistic function model feeding was found to be the optimal feeding strategy for SOS accumulation during *Flammulina velutipes* mycelia fermentation, where the maximum SOS accumulation reached (4.63 ± 0.24) mg/L, which is consistent with the predicted value.

Key words: Feeding Strategy, *Flammulina velutipes*, Soluble Organic Selenium

Introduction

Selenium is an essential nutrient with diverse physiological actions that include enhancement of immune function and reduction of cancer risk (Finley, 2006). There is evidence that less-overt Se deficiency can have adverse consequences for disease susceptibility and the maintenance of health (Rayman, 2000). The bioavailability of Se is closely correlated with its chemical forms. Organic Se sources, *e.g.* selenomethionine and Se-containing yeast, have a higher bioavailability than inorganic selenium sources, such as sodium selenite, and organic Se sources are often preferred because of their lower acute toxicity (Wang and Lovell, 1997; Rayman, 2000). Se-containing yeast has been the primary form of Se available for use as an organic Se source (Arpasova *et al.*, 2009; Juniper *et al.*, 2009). Edible mushrooms have been introduced as a new form of organic Se source (Ogra *et al.*, 2004; Yu *et al.*, 2009). While *Flammulina velutipes* is a good source of carbohydrates, proteins, fibers, essential amino acids, and minerals (Smiderle *et al.*, 2008), little attention has been paid to organic Se accumulation during the fermentation of *F. velutipes* mycelia. In fact, selenized *F. velutipes* mycelia will be a better form of organic Se because of their nutritional value and rapid growth in culture.

Fed-batch culture is a batch culture fed continuously or sequentially with substrate without the removal of fermentation broth, which is generally superior to batch processing and is especially beneficial when changing nutrient concentrations affect the yield or productivity (Miguel *et al.*, 2007). So, development of a suitable feeding strategy is critical in fed-batch cultivation. Exponential feeding is a simple method that allows cells to grow at a constant growth rate (Kim *et al.*, 2004), and exponential feeding of substrates has been widely applied during fermentation (Ding and Tan, 2006; Miguel *et al.*, 2007; Chen *et al.*, 2009). However, no constant specific growth rate of filamentous fungi can be assumed, when growth takes place in the form of pellets (Lejeune and Baron, 1998). Therefore, exponential feeding of Se is not applicable during the fermentation of *F. velutipes* mycelia. Here we present a feeding strategy for maximum soluble organic selenium (SOS) accumulation during fermentation.

[§] These authors contributed equally to this work.
Abbreviations: CCD, central composite design; RSM, response surface methodology; SOS, soluble organic selenium.

Response surface methodology (RSM) is an efficient statistical technique for the optimization of multiple variables to predict the best performance conditions with a minimum number of experiments (Gouda *et al.*, 2001). Recently, RSM and central composite design (CCD) have been applied to optimize the constituents of microbial fermentation media (Rodrigues *et al.*, 2006; Oskouie *et al.*, 2008). Here, we have investigated the effects of sodium selenite, glucose, and NH_4NO_3 on organic Se accumulation during the fermentation of *F. velutipes* mycelia by RSM.

Material and Methods

F. velutipes mycelia start culture

Potato dextrose broth (PDB) was prepared by cutting 200 g potatoes into 1-cm^3 pieces and boiling them in 500 mL of water for 20 min; then the extract was collected by filtration through gauze, followed by addition of 20 g glucose and water to 1 L total volume (Yokota *et al.*, 2010).

Strain F 39 of *F. velutipes* was from the Culture Collection of the Department of Microbiology of Huazhong Agricultural University (Wuhan, China). Actively growing mycelia obtained from potato dextrose agar (PDA; 10 g/L agar in PDB) were inoculated into 100 mL PDB and cultured in 250-mL shake flasks on a rotary shaker for 7 d at 20 °C. Sterile water was added to the flasks, and the mycelia were suspended in sterile water (1:100, w/v) for further experiments.

Optimization of the concentrations of glucose, ammonium nitrate, and sodium selenite

To investigate the optimal sodium selenite concentration for Se accumulation during fermentation, mycelia were inoculated in PDB containing various concentrations of sodium selenite (3.46, 6.92, 10.38, 13.84, and 17.3 mg/L) in a 250-mL shake flask. The PDB used for studying the effects of glucose on Se accumulation contained 10.38 mg/L sodium selenite, and 20, 30, and 40 g/L glucose, and the residual sugar was determined by the dinitrosalicylic acid (DNS) method (Lindsay, 1973). A PDB containing 10.38 mg/L sodium selenite, 200 g/L potato, and 20 g/L glucose was used to study the effects of ammonium nitrate (0, 1.0, 2.0, and 3.0 g/L NH_4NO_3) on SOS accumulation during fermentation.

Growth curve of F. velutipes mycelia in the fermentation broth

The mycelia were inoculated in 100 mL PDB and cultured at 20 °C in 250-mL flasks on a shaking table for 7 d, then dried at 60 °C and weighed.

SOS accumulation during fermentation

To investigate the SOS accumulation during fermentation under the presence of various sodium selenite concentrations, mycelia were inoculated in the optimal PDB (200 g/L potato, 30 g/L glucose, and 1.8 g/L NH_4NO_3) containing various concentrations of sodium selenite (6.92, 10.38, and 13.84 mg/L) in 250-mL flasks. The SOS accumulation rate [mg/(L d)] was determined at different culture times and Se concentrations during fermentation.

In this experiment, RSM and CCD were used to optimize the culture medium and the rate of SOS accumulation. The software Design-Expert 7.0.0 Trial (Stat-Ease Inc., Minneapolis, MN, USA) was applied in the experimental design, data analysis, and quadratic equation construction (Lu *et al.*, 2009). Independent variables were coded for statistical calculation:

$$x_i = \frac{X_i - X_0}{\Delta X_i}, i = 1, 2, 3,$$

where X_i is the experimental value, ΔX_i is the step change in X_i, X_0 is the midpoint of X_i, and x_i represents the coded values for X_i.

A second-order model was applied to find the optimal set of process conditions, and the relationship between variables and response was described according to the following equation:

$$y = \beta_0 + \sum_{i=1}^{k}\beta_i x_i + \sum_{i=1}^{k}\beta_{ii} x_i^2 + \sum_{i=1}^{k}\sum_{j}^{k}\beta_{ij} x_i x_j,$$

where y is the predicted response, x_i and x_j ($i < j$) are coded variables, β_0, β_i, β_{ii}, β_{ij} are regressive coefficients calculated from the experimental data by second-order multiple regression, and k is the number of factors.

The experimental data were statistically analysed by Fischer's statistical test for analysis of variance (ANOVA). The significance of each coefficient was analysed using the ANOVA test, and a P value (probability > F) less than 0.05 indicated that the model terms are significant.

Comparison of different feeding strategies

Sodium selenite was added to the fermentation broth according to different feeding strategies, *i.e.* logistic function model feeding fed-batch, constant rate feeding fed-batch, and batch culture, respectively.

Analytical methods

The soluble Se compounds in *F. velutipes* mycelia samples were extracted in 5 mL 10 mM Tris-HCl buffer, pH 8.0, with ultrasonication, and the homogenate was centrifuged (Chassaigne *et al.*, 2002). SOS compounds were determined by the method we reported previously (Ma *et al.*, 2009).

Amino acids were determined using a Hitachi amino acid analyzer, Model Hitachi L-8900 (Tokyo, Japan).

Results and Discussion

Optimal medium components for SOS accumulation

As shown in Fig. 1, growth of the mycelia was increasingly inhibited by sodium selenite with its concentration increasing from 3.46 to 17.3 mg/L. Incorporation of Se-amino acids (Se-Cys and Se-Met) is likely to affect the activity of enzymes (Schrauzer, 2002). While the content of SOS in the mycelia increased with the concentration of external sodium selenite, the dry weight of the mycelia decreased, and the SOS content in the fermentation broth reached a maximum value [(2.64 ± 0.064) mg/L] at 10.4 mg/L of sodium selenite (Fig. 1).

The SOS and the residual sugar contents increased with increasing initial glucose concentration, but in a non-proportional manner (Fig. 2). Therefore, an initial glucose concentration of 30 g/L was selected for the further experiments. The SOS content increased significantly with the NH_4NO_3 concentration increasing from 0 to 2.0 g/L, but did not increase further at 3 g/L, while the final pH value decreased steadily with increasing NH_4NO_3 concentration from 0 to 3.0 g/L (Table I), suggesting that NH_4^+ was utilized preferentially by the mycelia, and NO_3^- accumulated in the fermentation broth, leading to the pH decrease.

The amino acid composition of *F. velutipes* mycelia is presented in Table II. The contents of all amino acids increased greatly, with the exception of Cys, after addition of ammonium nitrate, with Met showing the highest relative increase. The results thus show that addition of NH_4NO_3 can promote the synthesis of amino acids in the mycelia. This is likely to be the reason why addition of NH_4NO_3 can effectively increase the SOS accumulation during *F. velutipes* fermentation. The results presented in Tables I and II indicate that NH_4NO_3 is an excellent nutrient to support Se accumulation in *F. velutipes* mycelia.

CCD was applied to find the appropriate dosages of sodium selenite and NH_4NO_3 and to predict the maximum SOS content in the culture

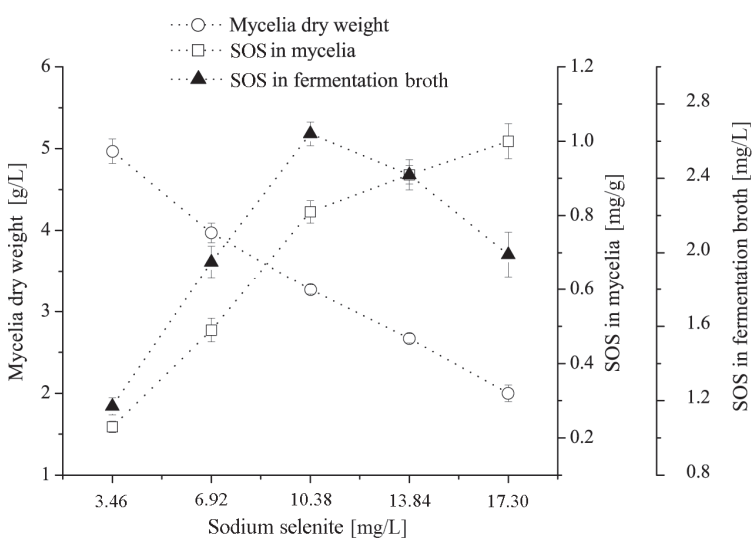

Fig. 1. SOS accumulation in mycelia as a function of sodium selenite concentration.

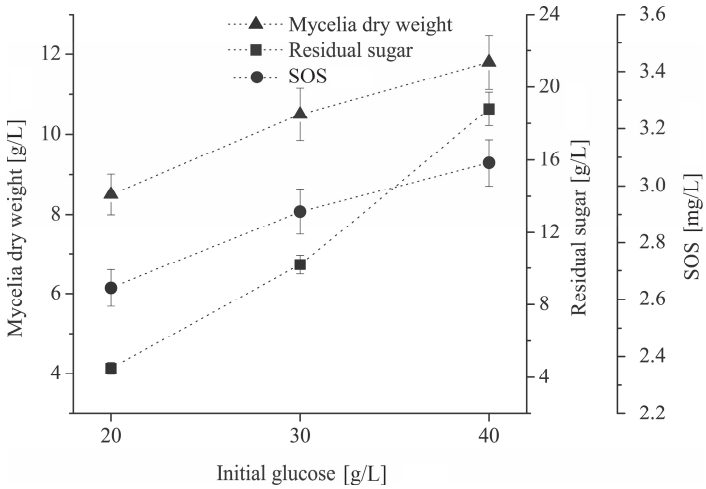

Fig. 2. SOS accumulation as a function of initial glucose concentration.

Table I. SOS accumulation in the fermentation broth of *F. velutipes* mycelia at different concentrations of NH_4NO_3.

NH_4NO_3 [g/L]	SOS [mg/L]	Final pH
0	2.64 ± 0.11	5.5 ± 0.2
1.0	2.98 ± 0.13	5.2 ± 0.2
2.0	3.21 ± 0.13	4.5 ± 0.3
3.0	3.18 ± 0.12	3.7 ± 0.3

broth. The variables and responses of SOS are listed in Table III.

Analyses of variance for the quadratic model of SOS accumulation are shown in Table IV. Values of "$P > F$" < 0.05 indicate, that the model terms were significant. In this case, X_1, X_2 and X_1^2, X_2^2 were significant model terms which indicated that changes in the concentrations of ammonium nitrate and sodium selenite affected the organic Se content directly. The F value of the X_1X_2 term was 0.5787, indicating that the interaction of ammonium nitrate and sodium selenite was not significant. The "lack of fit F value" of 4.10 implies that the "lack of fit F value" was not significant relative to the error. There is a 10.76% chance that a "lack of fit F value" this large could be due to noise. By ANOVA, the coefficient of determination (R^2) of the regression model was 0.9764 and the adjusted coefficient (Adj R^2) in the two models was 0.9595, which means a good agreement between the experimental and predicted values of the SOS content in the *F. velutipes* culture broth.

Table II. Amino acid composition of *F. velutipes* mycelia.

Amino acid	Composition (mg/g)*	
	a	b
Asp	16.6	24.9
Thr	9.0	14.0
Ser	13.6	20.9
Glu	25.5	40.2
Pro	7.6	12.9
Gly	9.7	15.2
Ala	12.4	20.2
Cys	4.1	3.8
Val	10.8	16.3
Met	7.9	17.2
Ile	8.2	12.8
Leu	14.0	22.0
Tyr	1.8	3.8
Phe	9.1	14.1
Lys	9.4	16.8
Arg	8.4	14.7
His	3.4	5.9
Total	171.5	275.5

* Dry weight, amino acid (mg)/mycelia (g).
a, Potato medium; b, potato medium + 2 g/L NH_4NO_3.

The results of CCD to predict the SOS content in the culture broth were fitted with a second-order polynomial function:

$$Y = -3.47 + 1.47X_1 + 1.0X_2 - 0.016X_1X_2 - 0.35X_1^2 - 0.043X_2^2, \quad (1)$$

where Y is the response, *i.e.* the SOS content (mg/L), X_1 is the concentration of sodium selenite

Table III. Central composite design for optimization of two variable experimental values for SOS.

Run	X_1	X_2	Y
1	13.8	2.00	3.08
2	10.4	1.50	3.38
3	5.49	1.50	1.98
4	10.4	1.50	3.41
5	10.4	0.79	3.12
6	13.8	1.00	2.86
7	15.6	1.50	2.89
8	10.4	1.50	3.49
9	10.4	1.50	3.42
10	6.92	1.00	2.35
11	10.4	1.50	3.32
12	6.90	2.00	2.68
13	10.4	2.21	3.46

X_1 and X_2 are the concentrations of sodium selenite (mg/L) and NH_4NO_3 (g/L), respectively. Y is the concentration of SOS (mg/L).

gistic function model of subsurface fungal growth has been applied to bioremediation (Wachenheim *et al.*, 2003). The growth curve of the mycelia was in agreement with the logistic model, and the growth properties of the mycelia can be represented by the equation in Fig. 4. In this equation, M is the mycelia dry weight (g/L), and t is the culture time (d):

$$M = 12.85 - \frac{13.0}{1 + \left(\frac{t}{3.96}\right)^{3.96}}, \; R^2 = 0.9963. \qquad (2)$$

Fig. 4 shows that the mycelia grew rapidly between days 3 to 5 of fermentation, likely concomitant with SOS accumulation. CCD was applied to find the appropriate sodium selenite concentration and the culture time to predict the maximal rate of SOS accumulation in the culture broth

Table IV. Analysis of ANOVA for the fitted quadratic polynomial model of SOS accumulation.

Source	Degree of freedom	Sum of squares	Mean square	F value	P value $P > F$
Model	5	2.58	0.52	57.89	<0.0001
X_1	1	0.60	0.60	67.60	0.0001
X_2	1	0.13	0.13	14.88	0.0062
X_1X_2	1	3.02E-003	3.02E-003	0.34	0.5787
X_1^2	1	1.84	1.84	206.32	<0.0001
X_2^2	1	0.053	0.053	5.90	0.0455
Residual	7	0.062	8.93E-003		
Lack of fit	3	0.047	0.016	4.10	0.1031
Pure error	4	0.015	3.83E-003		
Cor total	12	2.65			
C.V. % = 3.11		R^2 = 0.9764		Adj R^2 = 0.9595	

X_1 and X_2 are the concentrations of sodium selenite (mg/L) and NH_4NO_3 (g/L), respectively.

(mg/L), and X_2 is the concentration of NH_4NO_3 (g/L). From (1), the optimal conditions for sodium selenite and NH_4NO_3 are 11.2 mg/L and 1.85 g/L, respectively.

The effects of sodium selenite and NH_4NO_3, and their combinations, on the SOS content are shown in Fig. 3. The surface plots of yield indicate that the SOS content could not exceed 3.5 mg/L. In fact, the predicted maximum response and experimental values for SOS accumulation were 3.48 and (3.43 ± 0.13) mg/L (Table V), respectively.

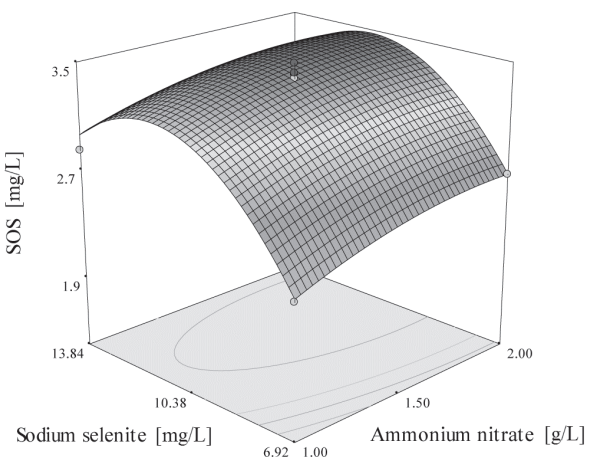

Fig. 3. Determination of the optimal concentration of NH_4NO_3 and sodium selenite for SOS accumulation.

SOS accumulation rate as a function of time and selenite concentration

The logistic function model is increasingly being used to describe microbial growth, and the lo-

Table V. Predicted and experimental values for SOS accumulation by the batch culture strategy.

Sodium selenite [mg/L]	NH_4NO_3 [g/L]	SOS [mg/L]	
		Experimental value	Predicted value
10.4	1.85	3.36 ± 0.14	3.43
11.2	1.85	3.43 ± 0.13	3.48
13.8	1.85	3.02 ± 0.11	3.19

(Fig. 5). The variables and responses of the SOS accumulation rate are listed in Table VI. Interestingly, the accumulation rate in Fig. 5 agrees well with the growth rate in Fig. 4, and it reached its maximum on day 4 (Fig. 5).

Analyses of variance for the quadratic model of the SOS accumulation rate are shown in Table VII. In this case, X_1, X_2 and X_1^2, X_2^2 were significant model terms which indicated that the sodium selenite concentration and culture time had a direct influence on the rate of SOS accumulation. The F value of the X_1X_2 term was 0.5171, indicating that the interaction between sodium selenite and culture time was not significant. The "lack of fit F value" of 3.98 implies that lack of fit was not significant relative to the error. There is a 10.79%

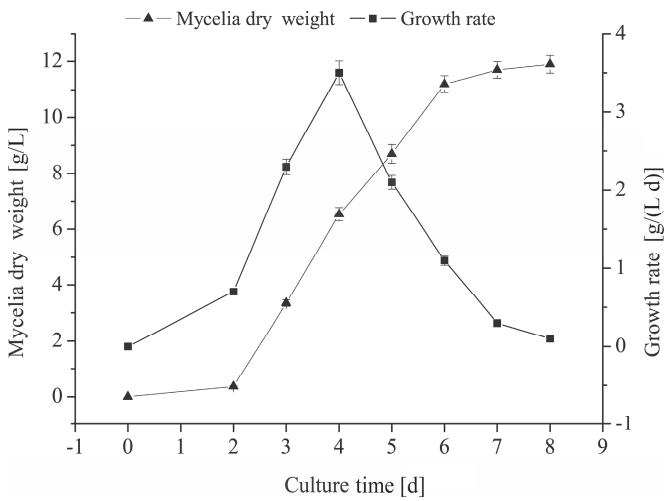

Fig. 4. Growth curve and growth rate of *F. velutipes* mycelia.

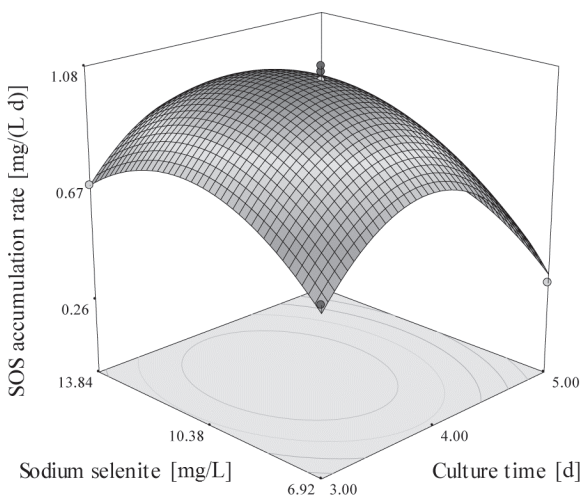

Fig. 5. SOS accumulation rate as a function of sodium selenite concentration and time.

Table VI. Central composite design for the SOS accumulation rate.

Run	X_1	X_2	Y
1	6.92	5.00	0.32
2	10.4	4.00	1.06
3	6.92	3.00	0.56
4	10.4	4.00	1.02
5	13.8	3.00	0.67
6	13.8	5.00	0.37
7	10.4	5.41	0.26
8	10.4	4.00	1.08
9	10.4	4.00	1.03
10	15.6	4.00	0.75
11	10.4	2.59	0.48
12	5.19	4.00	0.56
13	10.4	4.00	1.01

X_1 and X_2 are the concentration of Se(IV) (mg/L) and culture days, respectively. Y is the soluble organic selenium (SOS) accumulation rate [mg/(L d)].

Table VII. Analysis of ANOVA for the fitted quadratic polynomial model of the SOS accumulation rate.

Source	Degree of freedom	Sum of squares	Mean square	F value	P value $P > F$
Model	5	1.11	0.22	114.32	<0.0001
X_1	1	0.023	0.023	11.88	0.0107
X_2	1	0.091	0.091	46.82	0.0002
X_1X_2	1	9.0E-004	9.0E-004	0.47	0.5171
X_1^2	1	0.28	0.28	144.79	<0.0001
X_2^2	1	0.82	0.82	423.51	<0.0001
Residual	7	0.014	1.93E-003		
Lack of fit	3	0.010	3.38E-003	3.98	0.1079
Pure error	4	3.4E-003	8.5E-003		
Cor total	12	1.12			
C.V. % = 6.23		R^2 = 0.9879		Adj R^2 = 0.9793	

X_1 and X_2 are the concentrations of sodium selenite (mg/L) and culture days, respectively.

chance that a "lack of fit F value" this large could occur due to noise. By ANOVA, the coefficient of determination (R^2) of the regression model was 0.9879 and the adjusted coefficient (Adj R^2) in the two models was 0.9793, which means that the predicted SOS accumulation rate agrees well with the experimental accumulation rate in the culture broth.

Prediction of the SOS accumulation rate by CCD in the culture broth was fitted with a second-order polynomial function:

$$V = -6.17 + 0.381X_1 + 2.68X_2 - 0.0168X_1^2 - 0.343X_2^2, \quad (3)$$

where V is the response, *i.e.* the SOS accumulation rate [mg/(L d)], X_1 is the concentration of sodium selenite (mg/L), and X_2 is the culture time (d).

Optimal feeding strategy for SOS accumulation

Filamentous fungi may form pellets in submerged cultures, and earlier studies have demonstrated that the growth of fungal pellets is not exponential (Nielsen, 1996). The feeding strategy was according to the logistic function model. In (4), c is the concentration of sodium selenite (mg/L), t is the culture time (d), and A_1 and A_2 are constants (Wachenheim *et al.*, 2003):

$$c = A_1 - \frac{A_2}{1 + \left(\frac{t}{3.96}\right)^{3.96}} . \quad (4)$$

The relationship between SOS accumulation and accumulation rate can be described by the differential and integral equations

$$\mathrm{d}S = v\mathrm{d}t \quad S = \int_0^t v\mathrm{d}t + C.$$

In this equation, S is the SOS concentration (mg/L), v is the rate of SOS accumulation [mg/(L d)], t is the culture time (d), and C is a constant. The main SOS accumulation was completed between days 3 and 5 of fermentation (Fig. 4), so (2) was regarded as the integral function to describe SOS accumulation during fermentation. In this experiment, the logistic function model feeding strategy was compared with the constant rate feeding method, which was also used in fed-batch fermentation (Callewaert and De Vuyst, 2000). SOS accumulation and the different feeding strategies can be described by the following integral equations:

$$\begin{cases} S_1 = \int_0^6 (-6.17 + 0.381c + 2.68t - 0.168c^2 - 0.34t^2)\mathrm{d}t + C_1, \\ c = 1.73t; \end{cases} \quad (5)$$

$$\begin{cases} S_2 = \int_0^6 (-6.17 + 0.381c + 2.68t - 0.168c^2 - 0.343t^2)\mathrm{d}t + C_2, \\ c = 11.2 - \dfrac{11.4}{1 + \left(\frac{t}{3.96}\right)^{3.96}}. \end{cases} \quad (6)$$

C_1 (= 8.49) and C_2 (= 11.44) were obtained by the experimental values (S_1 = 3.18 ± 0.15; S_2 = 3.69 ± 0.19) subtracting the integral equation values, and equations (5) and (6) were described by the equations (7) and (8), respectively:

$$\begin{cases} S = \int_0^6 (-6.17 + 65.87c + 2.68t - 501.56c^2 - \\ \quad 0.34t^2)\mathrm{d}t + 8.49, \\ c = kt; \end{cases} \tag{7}$$

$$\begin{cases} S_2 = \int_0^6 (-6.17 + 65.87c + 2.68t - 501.56c^2 - \\ \quad 0.34t^2)\mathrm{d}t + 11.44, \\ c = A_1 - \dfrac{A_2}{1 + \left(\dfrac{t}{3.96}\right)^{3.96}}. \end{cases} \tag{8}$$

The experimental and predicted results, respectively, of equations (7) and (8) are presented in Table VIII. The batch culture reached maximum SOS accumulation at 11.2 mg/L sodium selenite (Table V), and the constant rate feeding fed-batch culture [equation (7)] and logistic function model feeding fed-batch culture [equation (8)] reached maximum SOS accumulation, when the terminal sodium selenite concentration was 17.3 mg/L (Table VIII). The optimal feeding strategy for SOS accumulation during fermentation of *F. velutipes* mycelia is the logistic function model feeding fed-batch culture [with maximum SOS accumulation of (4.63 ± 0.25) mg/L].

In conclusion, RSM and CCD were applied to optimize the composition of the fermentation broth, and the rate equations of SOS accumulation under different culture conditions were established. Based on the rate equations, the proposed integral equations can predict the SOS accumulation, and the logistic function model feeding fed-batch culture is an optimal culture strategy to accumulate SOS during fermentation of *F. velutipes* mycelia.

Table VIII. Predicted and experimental values for SOS accumulation by constant rate feeding and logistic feeding strategies.

Feeding strategy	Final concentration of sodium selenite [mg/L]	SOS [mg/L]	
		Experimental value	Predicted value
Logistic feeding	10.4	3.69 ± 0.18	-
	13.8	4.41 ± 0.25	4.78
	17.3	4.63 ± 0.24	5.04
	20.8	4.23 ± 0.21	4.68
Constant rate feeding	10.4	3.48 ± 0.15	-
	13.8	4.11 ± 0.17	4.62
	17.3	4.39 ± 0.21	4.96
	20.8	3.88 ± 0.20	4.50

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