Advance Journal of Food Science and Technology 5(2): 174-179, 2013
ISSN: 2042-4868; e-ISSN: 2042-7876

Submitted: September 14, 2012 Accepted: October 24, 2012 Published: February 15, 2013

Response Surface Methodology Used for Investigating Soluble Organic Selenium Accumulation in Yeasts

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Abstract: Selenium-enriched yeasts were usually regarded as an ideal organic selenium source. The study investigated the Soluble Organic Selenium (SOS) accumulation in three species of yeasts: Saccharomyces cerevisiae, Candida rugosa and Candida utilis. The effects of main factors on organic selenium accumulation were evaluated. The Response Surface Methodology (RSM) and Central Composite Design (CCD) were applied to optimize the dosage of main factors in fermentation medium. SOS content in the dry cells of *C. utilis* (1.31±0.065 mg/g) was higher than that of *S. cerevisiae* (1.20±0.057 mg/g) and *C. rugosa* (0.68±0.033 mg/g). However, as far as the productivity of per unit volume of broth was concerned, *Candida rugosa* also accumulated the most SOS (4.81±0.18 mg/L) among the three species of yeasts. Higher content of SOS in yeasts inhibited the cell growth and led to decrease of SOS per unit volume during yeasts fermentation. Perhaps, yeasts with lower content of SOS have the advantage of SOS accumulation per unit volume broth accumulation.

Keywords: Central Composite Design (CCD), optimal culture, organic selenium, Response Surface Methodology (RSM), selenium-enriched yeasts

INTRODUCTION

Selenium (Se) plays an important role in human health, particularly in relation to the immune response, antioxidant action and cancer prevention (Tapiero *et al*., 2003; Abdulah *et al*., 2005; Zeng and Combs, 2008). The bioavailability of selenium is closely correlated with its chemical forms. Organic selenium sources, selenomethionine and selenoyeast, had higher bioavailability than inorganic selenium sources in animal experiment (Wang and Lovell, 1997). In all cases described, selenomethionine is the largest single species and Se-yeast is capable of increasing the activity of the selenoenzymes and its bioavailability has been found to be higher than that of inorganic Se sources in all but one study and moreover intervention studies with Se-yeast have shown the benefit of this form in cancer prevention, on the immune response and on HIV infection (Rayman, 2004). Many selenium-containing compounds in selenium-enriched yeast were separated and determined, the distribution of Se-containing proteins in Se-enriched yeast has been refined and the relative molecular weight of SDS-soluble Se-containing proteins was determined between 10 000 and 100 000 (Chassaigne *et al*., 2002), which is an effective method of Se-containing proteins separated from selenium-enriched yeast.

The objective of this work is to evaluate the ability of soluble organic selenium accumulation of different species of yeast and choose the high selenium-enriched yeast to product organic selenium.

Here, we will seek for an effective way to accumulate organic selenium by investigating the effects of several factors on organic selenium accumulation during yeasts fermentation. Response Surface Methodology (RSM) is the one suitable method for identifying the effect of individual variables and for seeking the optimum conditions for a multivariable system efficiently and has been successfully applied to optimize fermentation media (Ratnam *et al*., 2005).

MATERIALS AND METHODS

Yeasts selection: Saccharomyces cerevisiae, Candida rugosa and Candida utilis were from China Center for Type Culture Collection (CCTCC), inoculated in 100 mL potato liquid medium (potato 20, glucose 2%, respectively) for activity. To investigate organic selenium accumulation of Saccharomyces cerevisiae,

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Candida rugosa and Candida utilis, three strains of yeasts were inoculated in the potato dextrose broth (PDB, potato 20%, glucose 2%) containing various concentrations of sodium selenite (0.04, 0.06, 0.08 and 0.1 mM) in 250 mL rotary shaker.

Optimization of the dosage of glucose, ammonium nitrate (NH_4NO_3) and sodium selenite: PDB (0.04, 0.06, 0.08, 0.10 mM sodium selenite, potato 20%, glucose 30 g/L) used for studying the effects of sodium selenite on selenium accumulation. Then liquid media (NH_4NO_3, 0, 1.0, 2.0 and 3.0 g/L, 0.08 mM sodium selenite, potato 20%, glucose 30 g/L,) used for studying the effects of ammonium nitrate on selenium accumulation in yeasts and the pH value of broth was determined respectively.

The Response Surface Methodology (RSM) and Central Composite Design (CCD) were applied to optimize the dosage of fermentation medium compositions (Himabindu *et al*., 2006; Rodrigues *et al*., 2006; Pan *et al*., 2008). In this experiment, RSM and CCD were used to optimize the NH_4NO_3 and Se concentration in fermentation medium (potato 20%, glucose 30 g/L). The software Design-Expert 7.0.0 Trial (Stat-Ease Inc., USA) was used for experimental design, data analysis and quadratic model building (Lu *et al*., 2009). For statistical calculation, independent variables were coded as:

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

where,

x_i = The coded values for X_i
X_i = The experimental value of variable
X_0 = The mid-point of X_i
ΔX_i = The step change in X_i

To find the optimum set of operating conditions and to describe the relationship between variable and response, a second-order model was applied 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 = Predicted response
x_i & x_j ($i<j$) = Coded variable
β_0, β_i, β_{ii}, β_{ij} = Regression coefficients obtained from the experimental results by the multiple regression and k is the number of factors

The experimental data were statistically analyzed Using the Fischer's statistical test for Analysis of Variance (ANOVA). The significance of each coefficient (linear or quadratic) was analyzed by the ANOVA test and *p*-value (probability>F) less than 0.05 denoted that the model terms are significant. The relation between the responses and the experimental levels of the variables in this work was illustrated by the fitted polynomial equation in the form of three-dimensional surface plots.

Analysis methods: The soluble organic selenium compounds in yeast samples were extracted in 5 mL Tris-HCl buffer 10 mM pH8.0 before ultrasonication and centrifugation (Chassaigne *et al*., 2002) and the soluble organic selenium compounds were determined by the method we reported previously (Ma *et al*., 2009)

The amino acids were determined using a Hitachi amino acid analyzer, Model Hitachi L-8900 (Tokyo, Japan). Amino acids compositions in mycelia, cultured in PDB with 3.0 g/L ammonium nitrate, were determined and mycelia cultured in PDB were used as the control.

RESULTS AND DISCUSSION

The inhibition of sodium selenite on the growth of yeasts and the inhibition were increased with concentration of sodium selenite from 0.04 to 0.10 mM and among three species of yeasts, *C. rugosa* possessed the highest level of tolerance to selenium, for Dry Cell Weight (DCW) of *C. rugosa* was the highest one among the three species of yeasts and as far as the productivity of per unit volume of broth was concerned, *C. rugosa* accumulated the most organic selenium among the three species of yeasts (Fig. 1). Se mainly into Se-Methionine (Se-Met) and incorporate it into protein in place of Methionine (Met), which as a rule does not significantly alter protein structure but may influence the activity of enzymes if Se-Met replaces Met in the vicinity of the active site (Schrauzer, 2000). This may be the reason why higher content of SOS in yeast lead to decrease of DCW per unit volume.

NH_4NO_3 was demonstrated to be a nutrient for organic selenium accumulation during selenoyeast fermentation. The SOS increased significantly with increasing NH_4NO_3 concentration from 0 to 3.0 g/L (Fig. 2). The amino acid composition of in yeasts was present in Table 1. It is interesting that almost the amino acids contents increased greatly after addition of NH_4NO_3. The results thus show that addition of NH_4NO_3 can promote the synthesis of amino acids in yeasts, which may be the reason why addition of NH_4NO_3 can effectively increase SOS accumulation in yeasts. On the other hand, with the increase of NH_4NO_3 concentration in broth, pH values decrease, which may be caused by yeasts preferentially using NH_4+. And perhaps, low pH values also inhibited SOS accumulation in yeasts when NH_4NO_3 concentration was increased to 4.0 g/L.

With sodium selenite concentration from 0.04 to 0.10 mM and NH_4NO_3 (1.0-4.0 g/L) used as nitrogen

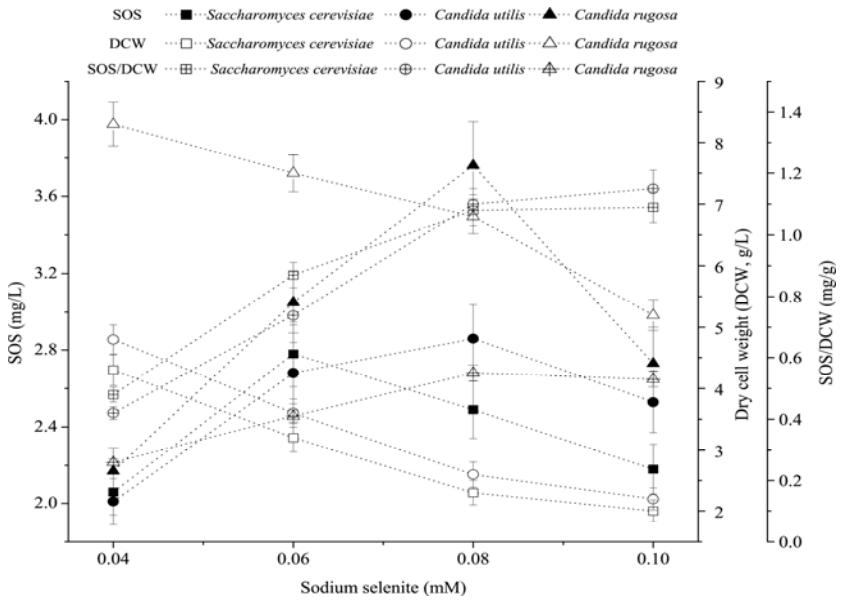

Fig. 1: Effects of selenite concentrations on soluble organic selenium synthesis in yeasts

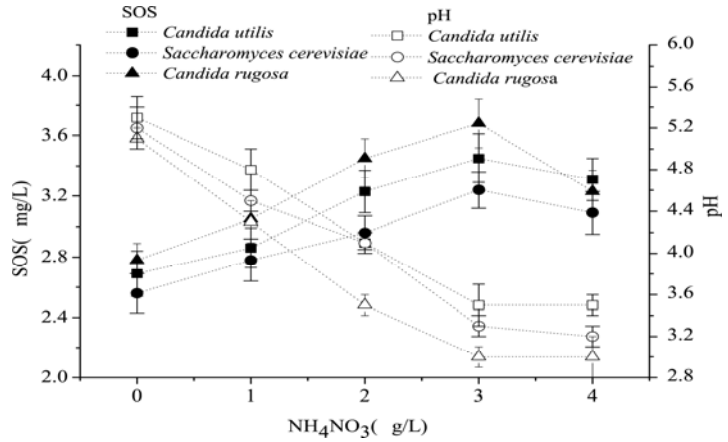

Fig. 2: Effects of NH_4NO_3 on soluble organic selenium accumulation in yeasts and pH value in fermentation broth

Table 1: Effect of NH_4NO_3 on amino acids content (mg/g) in yeasts

	C. rugosa		*S. cerevisiae*		*C. utilis*	
Amino acid	a	b	a	b	a	b
Asp	17.6	24.0	16.7	22.3	22.3	27.4
Thr	10.5	13.0	13.0	15.6	14.9	16.1
Ser	10.1	12.6	11.7	14.0	14.9	15.7
Glu	27.8	59.4	28.4	47.9	37.9	52.2
Gly	9.40	12.2	8.70	11.4	11.8	13.6
Ala	15.6	23.6	12.9	20.5	16.0	20.9
Val	10.2	13.5	11.0	14.1	14.4	16.7
Cys	1.30	1.60	1.30	2.00	1.80	2.40
Met	2.00	2.20	1.60	2.10	2.40	2.90
Ile	10.5	13.7	9.50	12.2	12.5	14.6
Leu	12.4	16.7	14.0	18.4	19.1	22.1
Tyr	5.50	7.30	6.30	8.50	9.40	10.9
Phe	8.10	11.0	8.60	11.2	11.7	13.4
Lys	11.0	16.6	12.5	17.0	16.5	20.1
His	3.70	5.50	3.70	4.80	4.90	5.80
Arg	7.50	10.5	7.90	10.9	10.9	13.1
Pro	8.30	10.5	4.20	5.40	4.90	8.60
Total	171.5	253.9	171.9	238.3	226.3	276.5

Table 2: Central composite design for optimal medium composite of soluble organic selenium synthesis in yeasts during fermentation

	C. rugosa			*C. utilis*			*S. cerevisiae*		
Run	X_1	X_2	Y	X_1	X_2	Y	X_1	X_2	Y
1	0.10	2.0	3.42	0.08	3.0	3.63	0.06	4.4	3.25
2	0.06	2.0	3.65	0.10	2.0	2.93	0.06	1.6	3.04
3	0.08	1.6	4.23	0.08	3.0	3.48	0.06	3.0	3.49
4	0.05	3.0	3.31	0.08	3.0	3.51	0.04	4.0	2.51
5	0.08	3.0	5.03	0.11	3.0	2.85	0.08	4.0	2.88
6	0.08	4.4	4.21	0.06	4.0	3.09	0.06	3.0	3.69
7	0.08	3.0	5.10	0.08	1.6	3.14	0.08	2.0	3.14
8	0.10	4.0	3.21	0.06	2.0	3.12	0.09	3.0	3.15
9	0.08	3.0	4.95	0.08	3.0	3.53	0.03	3.0	2.05
10	0.06	4.0	3.56	0.08	4.4	3.24	0.04	2.0	2.61
11	0.11	3.0	3.02	0.08	3.0	3.61	0.06	3.0	3.57
12	0.08	3.0	4.87	0.05	3.0	2.96	0.06	3.0	3.66
13	0.08	3.0	4.97	0.10	4.0	2.75	0.06	3.0	3.46

X_1 and X_2 are the concentrations of sodium selenite (mM) and NH_4NO_3 (g/L), respectively; Y: Soluble Organic Selenium (SOS) (mg/L) in broth

Table 3: Analysis of ANOVA for the fitted quadratic polynomial model of SOS synthesis in *C. rugosa*

Source	S.S.	df	M.S.	F value	p-value prob>F
Model	7.35	5	1.47	68.21	<0.0001
X_1	0.12	1	0.12	5.690	0.0486
X_2	0.013	1	0.013	0.630	0.4551
X_1X_2	0.0036	1	0.0036	0.170	0.6950
X_1^2	6.51	1	6.51	302.21	<0.0001
X_2^2	1.35	1	1.35	62.52	<0.0001
Residual	0.15	7	0.022		
Lack of fit	0.12	3	0.040	5.390	0.0687
Pure error	0.026	4	0.0067		
Cor total	7.50	12			
C.V.% = 3.57		R^2 = 0.9799, Adj R^2 = 0.9655			

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

Table 4: Analysis of ANOVA for the fitted quadratic polynomial model of SOS synthesis in *C. utilis*

Source	S.S.	df	M.S.	F value	p-value prob>F
Model	1.080	5	0.220	20.59	0.0005
X_1	0.041	1	0.041	3.960	0.0870
X_2	0.004	1	0.027	0.380	0.5566
X_1X_2	0.0004	1	0.0004	0.038	0.8505
X_1^2	0.850	1	0.850	81.03	<0.0001
X_2^2	0.300	1	0.300	28.38	0.0011
Residual	0.073	7	0.010		
Lack of fit	0.056	3	0.019	4.450	0.0916
Pure error	0.017	4	0.0042		
Cor total	1.150	12			
C.V.% = 3.19		R^2 = 0.9363, Adj R^2 = 0.8909			

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

sources, SOS accumulation in based on above experiments, respectively. Central Composite Design (CCD) was applied to find their appropriate dosage and predict maximum organic selenium content in yeasts. The variables and responses of SOS were listed in Table 2.

Analyses of variance for the quadratic model of SOS were shown in Table 3 to 5. Values of "p>F" <0.05 indicated model terms were significant. In Table 3 and Table 5, X_1, X_1^2, X_2^2 were significant model terms and in Table 4, only X_1^2, X_2^2 were significant model terms, which indicated change of sodium selenite concentration didn't always influence organic selenium accumulation significantly. In these cases, the F-value of X_2 and X_1 X_2 term were significant model terms, which indicated that the concentration of NH_4NO_3 and interaction between NH_4NO_3 and sodium selenite were not significant. Perhaps, the negative effect of high concentration of sodium selenite on cell growth counteracted the significance of sodium selenite to organic selenium accumulation and the interaction between NH_4NO_3 and sodium selenite. By ANOVA, the coefficients of determination (R^2) of the three regression model were 0.9799, 0.9363, 0.9441, respectively and the adjusted coefficients (Adj R^2) in the two models were 0.9655, 0.8909, 0.9041, respectively, which meant the agreements existed between the experimental values and the predicted values of organic selenium accumulation in yeasts.

The results of CCD to predict the organic selenium content in were fitted with second-order polynomial function:

Table 5: Analysis of ANOVA for the fitted quadratic polynomial model of SOS synthesis in *S. cerevisiae*

Source	S.S.	df	M.S.	F value	p-value prob >F
Model	2.770	5	0.550	23.63	0.0003
X_1	0.750	1	0.750	32.19	0.0008
X_2	0.0005	1	0.0005	0.021	0.8883
X_1X_2	0.0064	1	0.0064	0.270	0.6173
X_1^2	1.800	1	1.800	76.93	<0.0001
X_2^2	0.390	1	0.390	16.60	0.0047
Residual	0.160	7	0.023		
Lack of fit	0.120	3	0.041	4.000	0.1066
Pure error	0.041	4	0.010		
Cor total	2.930	12			
C.V.% = 4.91		R^2 = 0.9441, *Adj* R^2 = 0.9041			

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

Table 6: Predicted and experimental values for SOS in yeasts of NH_4NO_3 and selenite

			SOS (mg/L) in broth		
	Selenite (mM)	NH_4NO_3 (g/L)	Predicted values	Experimental values	SOS (mg/g) in dry yeasts cell
C. rugosa	0.078	2.97	4.93	4.81±0.18	0.68±0.033
C. utilis	0.079	2.94	3.62	3.65±0.12	1.31±0.065
S. cerevisiae	0.069	2.90	3.55	3.52±0.15	1.20±0.057

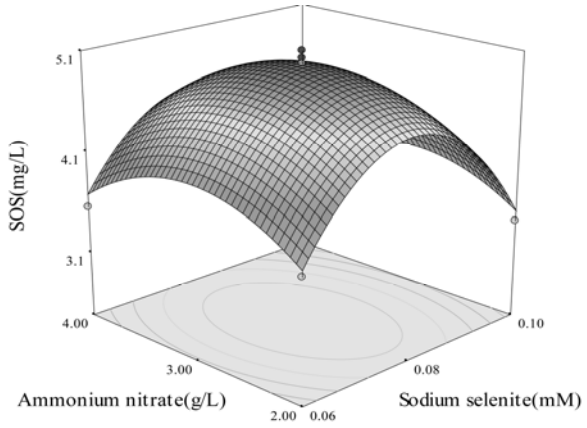

Fig. 3: The optimal concentration of NH_4NO_3 and selenite for SOS synthesis in *C. rugosa*

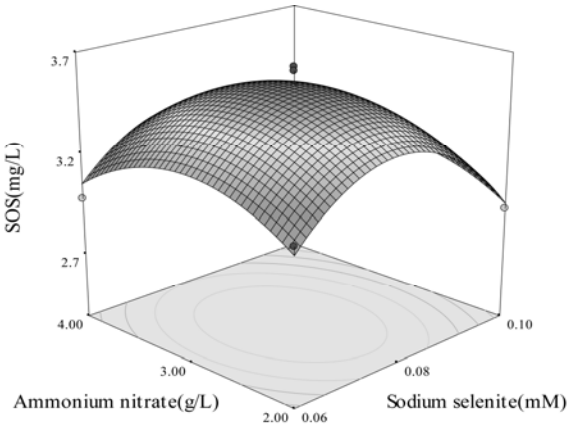

Fig. 5: The optimal concentration of NH_4NO_3 and selenite for SOS synthesis in *S. cerevisiae*

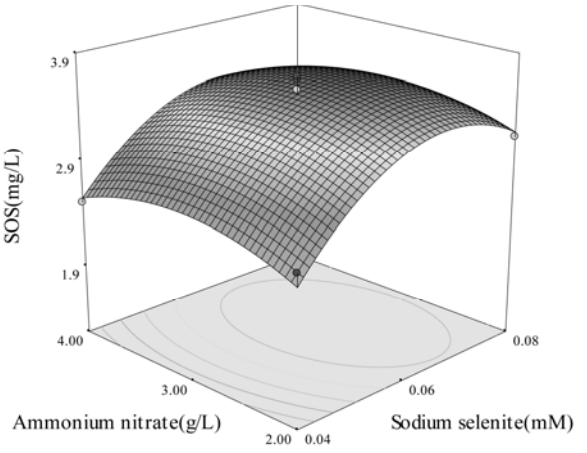

Fig. 4: The optimal concentration of NH_4NO_3 and selenite for SOS synthesis in *C. utilis*

$$Y_{C.\ rugosa} = -14.2 + 385.36X_1 + 2.72X_2 - 1.5X_1X_2 - 2419.06X_1^2 - 0.44X_2^2$$

$$Y_{C.\ utilis} = -3.66 + 137.55X_1 + 1.25X_2 - 0.5X_1X_2 - 872.81\ X_1^2 - 0.21X_2^2$$

$$Y_{S.\ cerevisiae} = -4.39 + 174.01X_1 + 1.53X_2 - 2.0X_1X_2 - 1272.19\ X_1^2 - 0.24X_2^2$$

where,

Y = The response, the soluble organic selenium content (mg/L)
X_1 = The concentrations of sodium selenite (mM)
X_2 = The concentrations of NH_4NO_3 (g/L)

The relation between SOS in yeasts and sodium selenite, $NH_4\ NO_3$ in media were shown in Fig. 3 to 5. The surface plots of yield indicated that the SOS accumulation were different in the three species of yeasts. By the second-order polynomial functions, the optimal conditions for sodium selenite and NH_4NO_3 and the predicted values and the experimental values of SOS accumulation in the three species of yeasts were obtained in Table 6. The experimental values for SOS

accumulation were near to the predicted values. The results might provide a reference for the production of SOS in yeasts. The SOS content in the dry cells of *C. utilis* (1.31±0.065 mg/g) was higher than that of *S. cerevisiae* (1.20±0.057 mg/g) and *C. rugosa* (0.68±0.033 mg/g). The content of amino acids, especially content of Cysteine (Cys) and Met in *C. utilis* is highest among the three species of yeasts (Table 1). From biosynthesis process of Se-Met, inorganic Se compounds can form Se-Met by selenocysteine (Se-Cys) pathway in the presence glutathione (Rayman *et al*., 2008) and the content of glutathione in *C. rugosa* is lower than that in *C. utilis* and *S. cerevisiae* significantly (Ma *et al*., 2010). These are the possible reasons why the higher content of Cys and Met can enhance SOS accumulation in yeasts and the SOS content in *C. rugosa* is lower than that in *C. utilis* and *S. cerevisiae*. On the other hand, as far as the productivity of per unit volume of broth was concerned, *C. rugosa* was able to accumulate the most organic selenium (4.81±0.18 mg/L) among the three species of yeasts. Perhaps, less SOS accumulation in *C. rugosa* bring about slight inhibition of yeast growth, which leads to higher SOS productivity of per unit volume of broth

CONCLUSION

Yeasts had the different SOS accumulation abilities and a few ways were found to increase the content of SOS in yeast. However, higher content of SOS in yeast inhibits the cell growth and possible leads to decrease of SOS per unit volume during yeasts fermentation. Therefore, yeasts with lower content of SOS have the advantage of SOS accumulation per unit volume broth.

ACKNOWLEDGMENT

The authors are grateful to Dr. Ming Ma of Technology Center of Ningbo Entry-Exit Inspection and Quarantine Bureau for the help of amino acids analysis.

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