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An Experimental Design for Production of Selenium-Enriched Yeast

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Abstract: Selenium (Se) enriched yeast, produced by growing of *Saccharomyces cerevisiae* in Se rich media, is a recognized source of organic food-form of Se. Effects of culture conditions (temperature, fermentation time, initial pH value, shaking speed, as well as time and concentration of Se added to medium) on the bioaccumulation of Se in yeast were investigated by Plackett-Burman design in this paper. Fermentation was carried out at different temperature (28-30°C), initial pH value (4.5-5.8), shaking speed (130-160 rpm), fermentation time (24-48 h), size of inoculums (30-60 g/l), Se concentration (15-25 µg/ml) and time of Se addition (0-9 h). The results showed total Se accumulation and organic Se formation ranges of 107.9 to 287.6 mg/kg and 93.27 to 269.05 mg/kg, respectively. The most suitable conditions for total and organic Se incorporation in yeast were: addition of sodium selenite 25 µg/ml, at 9 h after inoculation, inoculums size of 30 g/l, temperature 28°C, initial pH value 5.8, shaking speed 130 rpm and incubation time of 48 h.

Key words: Selenium-enriched yeast · Se incorporation · Plackett-Burman design · Biotransformation

INTRODUCTION

The trace mineral Se is a crucial nutrient for human health and its deficiency is considered to be important in various types of cancer. This element is a component of some important selenoproteins and enzymes required for main functions in organisms as antioxidant defense, reduction of inflammation, thyroid hormone production, DNA synthesis, fertility, reproduction [1] and killing cancer cells (by reducing the blood supply to them) [2]. So adequate dietary intake of Se is essential. The Department of Health, UK advised in 1991 that suitable average intakes of Se for an adult man and woman are 75 and 60 µg/day, respectively. At the same time, upper intake level for adults is 300 µg Se/day [3]. Recommended daily allowance of Se in USA is 55 µg/day [4]. Extradietary consumption of Se in the form of a nutritional supplement at the dosage less than 200 µg/day is considered safe for an adult of average weight [4, 5] and also is recommended to prevent cancer of the colon [6, 7].

In some parts of the world where se is insufficiently available to plants, se-deficiency diseases have been identified, such as Keshan disease, an endemic cardiomyopathy found in the North East of China [8] that formerly caused many deaths. Supplementation with Se has greatly reduced the incidence of the condition [9]. Supply of the Se enriched food, especially, Se enriched biomass (yeast or bacterial) with organic forms of this mineral is one efficient way to overcome Se deficiency [10, 11].

Yeasts contain high amount of protein and in compare to plant sources they can incorporate Se by replacement of S in proteins. Yeast can utilize soluble sugars and organic acids to produce biomass with high protein [12-14] and its production is easy to manage. By growing in Se-enriched media, yeast can accumulate large amounts of Se and incorporate them into organic Se-containing compounds, mainly Se-Met [15, 16]. Na_2SeO_3 can be bio-transformed to organic form and being absorbed by the yeast [17]. By this process the inorganic selenite as a low bioavailable toxic component can be converted to safer highly bioactive species with improved nutritional properties. *Saccharomyces cerevisiae* is only yeast strain that has been used by manufacturers for production of Se-enriched yeast [18]. CSI (Cypress Systems, Inc) report that mentioned yeast can be considered to be GRAS [19].

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FDA has defined several food categories intended for addition of Se-enriched yeast, along with the anticipated use levels and maximum numbers of daily servings. These intended foods includes baked products, beverages (nonalcoholic), breakfast cereals, gram products and pastas, milk products, processed fruits, fruit juices, processed vegetable juices, soups and soup mixes, commercial and medical foods. An adult human exposure to Se-yeast that provides up to 0.15 mg/day of Se is considered to be generally recognized as safe [19].

Although batch, fed-batch and continuous culture systems are applied for production of Se-enriched yeast, but batch strategy is most common. Several culture conditions of Se enriched yeast production including source of inorganic Se, concentration of Se, addition protocol of Se, base medium, pH, temperature, shaking speed, aeration, inoculums size, incubation time on yield of incorporated Se, (µg incorporated Se/g yeast) and yield of biomass production (g produced biomass/l medium) have been reported [7, 14, 17, 20-22] and summarized elsewhere [23]. Also several culture conditions of Se-enriched yeast production has been reported [23]. None of these researches simultaneous screening of variables had not been described.

A well defined statistical design of experiments (DOE) is necessary for optimization of culture parameters of Se-enriched yeast and to get more information through conducting fewer measurements. The Plackett-Burman design (PBD) has been frequently used for screening process variables that make the greatest impact on a process [24]. This small, efficient and powerful design is widely applicable and especially well suited for biotechnology research and developments [25-27]. The usefulness of the design lies in the fact that in determining the effects of one variable, the net effects of changing other variables cancel out so that the effect of each variable on the system can be independently determined.

In this study the application of PBD as a screening design for evaluation of relative importance of seven process variables including medium components and culture conditions on cell growth and Se incorporation by *S. cerevisiae* was investigated.

MATERIALS AND METHODS

Materials: The *S. cerevisiae* was purchased from Lesaffre yeast Company, Germany. The sodium selenite used for the enrichment of *S. cerevisiae* was purchased from Sigma Chemical Company. The All chemicals for enrichment of culture were of analytical grade and purchased from Merck Company.

Fermentation Conditions: The bakery-used strain of *S. cerevisiae* (active dried yeast) was cultivated to produce the Se-enriched yeast biomass. Different amount of yeast was cultivated for 24, 48 h in 100 ml of media with different concentrations of sodium selenite in Erlenmeyer flasks and shaken in an incubator at different temperature, shaking condition and pH medium. Sterile sugarcane molasses (40% brix) used as a base medium. The media had the following composition, g/l: zinc chloride 1.33, magnesium chloride 1.16 and thiamine 0.2; calcium pantothenate 0.025; sodium citrate 15; and sodium glutamate 10. Se was added to the sterile media in a form of Na_2SeO_3. The Se concentrations in the media were 15 and 25 µg/ml. In the course of cultivation, yeast growth was followed by measuring the density of the growing cells. The optical densities of withdrawn samples were measured by Spectrophotometer (Spectronic 70 Bausch and Lomb) at wave length of 540nm.

Determination of Se Content in Yeast: The yeast cells were separated after cultivation from liquid media by centrifugation (3000 ×*g*, 5min) and washed three times by deonized water for the removal of Se adsorbed on the cell surface. The cells were dried under vacuum to a constant weight. The Se determination was carried out according to the graphite furnace atomic absorption spectroscopy (GF-AAS) method. 0.2 g dried samples were digested (105°C, 20 min) with 10 ml of concentrated HNO_3 in a digestive flask. Then the solution was cooled and for accomplishing the digestion, the solution was heated with 2 ml of concentrated HCl (80°C, 10 min). After cooling, the solution was filtered and made a constant volume with ultra-pure water. The digested product was used for total Se determination. For measurement of inorganic Se, the suspension of biomass in ultra-pure water was extracted in boiling bath for 1 h and made a constant volume. Then the mixture was centrifuged at 8300 ×*g* for 15 min and the supernatant liquor was filtrated. Organic Se yield was calculated from the difference between the total and inorganic Se yield.

Experimental Design

Plackett-Burman Design (PBD): The variables to be evaluated include some culture conditions (i.e., temperature, pH, speed shaking, inoculums size,

Table 1: Variables to be monitored in PBD for biomass yield, total Se accumulation and organic Se formation

High Level (+)	Low levels(-)	Variables
48	24.0	A Time (h)
9	0.0	B Addition time of Se source (h)
25	15.0	C Se concentration (µg/ml)
60	30.0	D Inoculums size (g/l)
160	130.0	E Speed shaking (rpm)
5.8	4.5	F pH
30	28.0	G Temperature (°C)

Table 2: Eight-trial PBD to study of seven factors in biomass yield, total Se accumulation and organic Se formation

Coded setting for factors							
G	F	E	D	C	B	A	Trial
+	+	+	-	+	-	-	1
+	+	-	+	-	-	+	2
+	-	+	-	-	+	+	3
-	+	-	-	+	+	+	4
+	-	-	+	+	+	-	5
-	-	+	+	+	-	+	6
-	+	+	+	-	+	-	7
-	-	-	-	-	-	-	8

fermentation time) and Se source concentration and time of Se addition. Table 1 shows variables and their two levels which were used in this study. Table 2 shows selected experimental variables and a PBD for conducting eight experimental trials. The elements, + (high level) and - (low level) represent the two different levels of the independent variables examined. The layout of the matrix, given in Table 2, shows that each variable is equally at a high and a low level four times in each column.

RESULTS AND DISCUSSION

Analysis of PBD Experiments: The basic equation set up for the design was as follows. The coefficients for the seven variables were determined by:

$$A_1 = \frac{1}{N}\sum_{i=0}^{N} X_i.K_i$$

where Ai = coefficient values, X_i = experimental yield, Ki = coded value of each variable corresponding to the respective experimental yield X_i and N = number of experiments. Table 3 gives a comparison of the experimentally determined Se yield and biomass production to those predicted by solving the above equation, where predicted yield is given by:

$$Y_t = \sum_{i=0}^{N} A_i.K_i$$

Table 3: The results of seven process variables on biomass yield, total Se accumulation and organic Se formation by PBD

Organic Se formation (mg/kg)	Total Se accumulation (mg/kg)	Biomass yield (g/l)	Run No.
213.82	232.55	47.59	1
174.77	181.20	72.40	2
222.75	232.55	38.83	3
269.05	287.65	53.69	4
215.40	224.05	65.77	5
139.92	145.20	98.37	6
93.27	107.90	98.37	7
123.32	130.55	46.32	8

For i = 0, a dummy level of +1 was used and the coefficient obtained was called A_0. The standard error was determined as the sum of the squares of the difference between the experimental and predicted yield for each run. The estimated error is given by:

$$S_b = \sqrt{\frac{S_e^2}{N}}$$

The student's t-test was performed to determine the significance of each variable employed (t-value = coefficient/S_b). The statistics significance was evaluated using Student's t-test and $P < 0.05$ was taken as significant.

Experiment Results: In this experiment, the yeast Se enrichment possibility was studied. The influences of many factors upon the amount of Se in yeast were determined. In Table 2 the principal preparation conditions are shown. Table 3 shows the total Se, organic Se and biomass yields in samples. Also The correlation of each process variable on system responses include total Se accumulation, organic Se formation and biomass yield have been shown in Figure 1.

In Table 4 and 5 the effects of 7 factors in biotransformation of total Se and organic Se formation in yeast have been shown. It should be understood that Se can accumulate in yeast cells in organically bound and/or inorganic form and in certain circumstances elementary Se is formed, which gives a reddish color to the yeast cells. Color of yeast biomass grown in the media in all samples was drab which indicated that elemental Se that accumulated in the biomass was in low level. The result showed that concentration of Se sources had the most important factor in total Se accumulation (P=0.005) and organic Se formation (P=0.006) in yeast. Content of total Se and organic Se in yeast biomass correlated positively with its concentration in the medium. Second significant factor for both total Se accumulation (P=0.007)

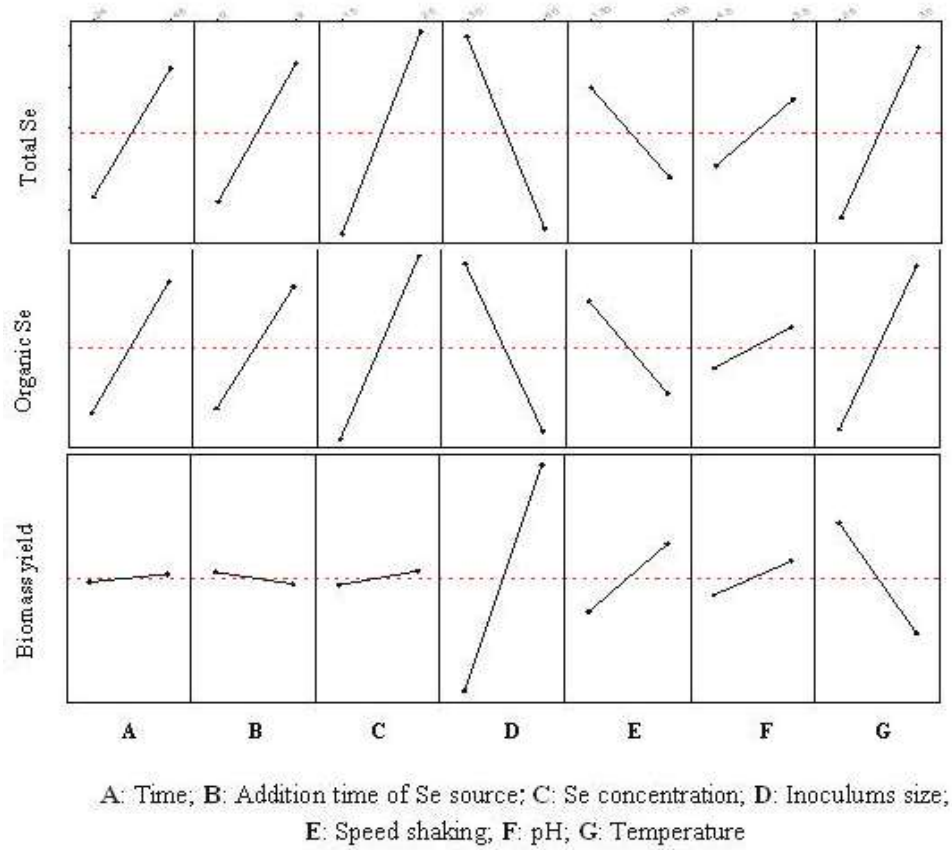

Fig. 1: The correlation of each seven process variables on system responses include total Se accumulation, organic Se formation and biomass yield

Table 4: The influences of seven process variables on total Se accumulation (mg/kg)[a] by PBD

P	*T*	Coef	Effect	Term
0.000	24.75	192.71		Constant
0.041	2.43	18.94	37.89	A
0.031	2.61	20.33	40.66	B
0.005	3.81	29.66	59.31	C
0.007	-3.61	-28.12	-56.24	D
0.130	-1.69	-13.16	-26.31	E
0.252	1.24	9.62	19.24	F
0.013	3.20	24.88	49.76	G

[a]Standard deviation coefficient was calculated as 7.787

Table 5: The influences of seven process variables on organic Se formation (mg/kg)[a] by PBD

P	*T*	Coef	Effect	Term
0.000	23.87	181.54		Constant
0.030	2.64	20.08	40.17	A
0.040	2.44	18.58	37.16	B
0.006	3.68	28.01	56.02	C
0.010	-3.38	-25.70	-51.39	D
0.101	-1.85	-14.10	-28.19	E
0.439	0.81	6.19	12.38	F
0.011	3.31	25.15	50.29	G

[a]Standard deviation coefficient was calculated as 7.607

and organic Se formation (P=0.010) was inoculums size of yeast. Both responses correlated negatively with inoculums size of yeast. In low level of inoculums size total Se accumulation and organic Se formation were increased. Similar results had reported by other researchers [28]. Temperature was the next significant factor for both total Se accumulation (P=0.013) and organic Se formation (P=0.011). Increased temperature causes more Se incorporation and more organic Se formation. Time of Se source addition is another significant factor in total Se accumulation (P=0.031) and organic Se formation (P=0.040). The results show that addition of Se sources in logarithmic phase of growth cycle (9 h) enhances Se accumulation also production of organic forms. Also by increasing incubation time, incorporation of Se and organic forms production were intensified. Content of Se in yeast biomass (P=0.041) and organic Se formation (P=0.030) correlated positively with fermentation time. pH could affect cell membrane function, cellular morphology and structure, the uptake of various nutriments and product biosynthesis. At high

Table 6: The influences of seven process variables on biomass yield (g/l)[a] by PBD

P	*T*	Coef	Effect	Term
0.000	94.74	65.181		Constant
0.360	0.97	0.668	1.337	A
0.178	-1.48	-1.015	-2.030	B
0.126	1.71	1.174	2.348	C
0.000	27.00	18.572	37.145	D
0.000	8.16	5.612	11.225	E
0.003	4.15	2.857	5.715	F
0.000	-13.09	-9.008	-18.017	G

[a] Standard deviation coefficient was calculated as 0.688

level of pH (5.8) yield of biomass increased and consequently incorporation of Se and formation of organic Se wereenhanced.

The results of other research [17] indicate that the dissolved oxygen concentration during the growth phase and the specific Se consumption rate are reflected in the inorganic Se content of the product. So by increasing dissolved oxygen Se incorporation decreased and inorganic content increased also the biomass color changed from drab to pink. The results showed that inorganic content of Se in media was not high enough to cause pink color (more than 25%). The samples had drab color and inorganic percent of samples were approximately 6%.

Table 6 presents influence of yeast biomass production by seven process variables include culture conditions (i.e., temperature, pH, speed shaking, inoculums size and fermentation time), Se source concentration and time of Se addition. The results showed inoculum sizes, temperature, shaking speed and pH value have significant impact on yeast growth respectively.

Although growth of yeast inhibited by the presence of Se in medium [7] but in this research Se concentration had not any significant effect on biomass yield. Also time of Se addition was not a significant variable. It showed that *S. cerevisiae* resisted to Se concentration of medium. Furthermore inoculation of yeast cells having a certain maturation growth into culture medium is the another reason for resistance of yeast. In this case, amino acids and proteins are synthesized relatively quickly and can assimilate and incorporate Se efficiently in the yeast metabolism. Also, Se toxicity had a relatively little effect on grown yeast cells whereas a growth cell restriction could happen in the case of using a microbial culture as inoculums. Moreover Nagodawithana and Gutmanis [29] developed a procedure for the propagation of a food-grade Se-enriched yeast and showed that under conditions of sulfur deficiency, Se could replace S because of their close similarities in yeast metabolism. So yeast assimilates Se as a replacement for S deficiency [29].

CONCLUSION

Statistically based experimental designs have proven to be valuable tools in optimizing culture conditions [26, 27]. One of the advantages of applying multi-factorial experiments is that such an approach considers the interaction between the non-linear behaviors of the responses in short experiments. In the present study, PBD was used to test the relative importance of culture conditions and process variables on cell growth and the production of Se-enriched yeast. Using various fermentation conditions include (i.e., temperature, pH, speed shaking, inoculums size, fermentation time) and Se source concentration and time of Se addition results in total Se accumulation in the range of 107.9-287.65 mg/ kg and inorganic Se yield approximately 6%.

The optimized values of the variables for production of Se-enriched yeast were as follows:

Se concentration, 25 µg/ml; addition time of Se source, 9 h; inoculums size, 30 g/l; shaking rate, 130 rpm; fermentation time, 48 h; temperature, 28°C; and pH, 5.8 that have maximum Se incorporation. These values can be selected as the best condition for further studies as an effective fermentation process for Se yeast supplementation. Also application of produced Se yeast in bread will be examined to access 5µg Se/serving (which for bread is 30 g) as defined safe daily usage.

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