Production of Organically Bound Selenium Yeast by Continuous Fermentation†

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This paper describes a protocol for incorporation of sodium selenite or sodium selenate into *Saccharomyces cerevisiae* biomass by continuous fermentation in a medium with minimal sulfur and methionine concentrations. Selenium incorporation was followed by atomic absorption analysis and methylene blue reduction time (MBRT). Continuous fermentation at 0.2 h^{-1} dilution rate and sodium selenite addition gradient up to 0.69 g/L of Na_2SeO_3 yielded 1.89 g/L of biomass with 1904 μg of selenium/g of dry biomass. However, MBRT was 0.1 min, which indicated that the majority of selenium was in the inorganic form. On the other hand, continuous fermentation at 0.2 h^{-1} dilution rate and sodium selenate gradient up to 0.28 g/L of Na_2SeO_4 yielded 0.76 g/L of dry biomass with 687 μg of selenium/g of dry biomass, and MBRT was 26 min, which indicated a high concentration of organically bound selenium. Overall, the results indicate a Se/S ratio of 3.9:1 and a dry biomass/Se ratio of 5.5:1 as optimal for continuous production of organically bound selenium.

Keywords: *Selenium; yeast; Saccharomyces cerevisiae; fermentation*

INTRODUCTION

At high concentrations, selenium is toxic and affects the central nervous system (Diaz-Alarcon et al., 1994). However, at low concentration, selenium is an essential element for animal and human diets (Kiffney and Knight, 1995). Selenium was identified as a part of cellular glutathione peroxidase, which provided evidence for selenium involvement in other metabolic processes (Schwarz and Foltz, 1957). Selenium deficiency has been associated with loss of hair pigment and macrocytosis in intravenously fed children (Foster and Sumar, 1997). Also, selenium deficiency was extensively studied on Keshan and Kashin-Beck diseases in China (Foster and Sumar, 1997). Recently, researchers found evidence for selenium as a cancer-protective agent (Combs, 1997). Because selenium affects the immune system, it has been found that selenium has a profound effect on survival in HIV-infected patients (Bologna et al., 1994).

Therefore, selenium must be provided to humans and animals as a part of nutritional intake. Selenium levels in food product depend on selenium levels in the soil where the agricultural product is grown (Ari et al., 1991). Selenium-supplemented diets are required for areas where soils contain negligible amounts of selenium. Because selenium and sulfur have similar chemical properties, the oxyanions selenite or selenate seem to be good candidates for fortification, because they can be regarded as analogues of sulfite and sulfate. Therefore, the U.S. Food and Drug Administration (FDA) approved sodium selenite and sodium selenate for supplementation at 0.3 ppm of Se in animal feed (FDA, 1987). However, it has been found that inorganic selenium may not be as bioavailable as organically bound selenium (Mahan and Kim, 1996). Also, retention of organically bound selenium is higher than that of inorganic selenium (Foster and Sumar, 1997). Grains are the major sources for organically bound selenium largely as selenomethionine (Levender, 1986; Ouyang and Wu, 1988). To produce organically bound selenium, selenium can be incorporated into organic seleno amino acid analogues; the principal one is selenomethionine (Kelly and Power, 1998; Stadtman, 1990). Nagodawithana et al. (1985) reported production of selenium yeast in fed-batch fermentation. However, they provided no explanation for selenium salt selection, for selenium salt feeding concentrations, and for minimum sulfur concentration.

In this study, we employed a continuous fermentation system to evaluate sodium selenite and sodium selenate incorporation into *Saccharomyces cerevisiae* as organically bound selenium. We identified the best ratio of selenium to sulfur for optimal incorporation of selenium. Because of the toxic nature of these compounds, several adapted strains were recovered and freeze-dried for future research.

MATERIALS AND METHODS

Microorganism. *S. cerevisiae* (ATCC 26787) was maintained as a freeze-dried culture and as a working culture in broth stored at 4 °C. The culture medium contained 20 g of glucose, 6 g of yeast extract (Ardamine-Z, Champlain Industries, Inc., Clifton, NJ), 0.23 g of $CaCl_2 \cdot 2H_2O$, 3.2 g of NH_4Cl, 1.6 g of $MgCl_2 \cdot 6H_2O$, and 1.5 g of KH_2PO_4 per liter of deionized water. Monthly serial transfers of working culture were performed to maintain viability.

Continuous Reactor Design. A New Brunswick Bioflo 3000 benchtop fermentor (Edison, NJ) equipped with pH, temperature, agitation, and antifoam controls was employed. The 1.2 L vessel with a working volume of 850 mL was equipped with air-in and -out ports, alkali and media addition ports, and effluent side ports. Liquid breaks were placed on

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the medium feed lines into the reactor and the medium effluent line out of the reactor to prevent back flow of cells from the fermentor into the medium feed and to prevent bioreactor contamination, respectively. Fermentation was performed at 30 °C and pH 5.5 with addition of 4 N NaOH. Foam was controlled with 8% Antifoam A (Sigma Chemical Co., St. Louis, MO). Aeration was maintained at 1 air volume/working volume per minute (vvm). Purity of the culture in the reactor was periodically checked microscopically throughout the fermentation.

Stock Media Preparation. Bulk media (100 L) were sterilized in a B.B. Braun U-100 fermentor (Allentown, PA) with constant agitation (220 rpm) at 121 °C for 30 min. A sterile KH_2PO_4 solution was added aseptically to medium in the fermentor before dispensing. After the pH had been adjusted to 5.5 with 4 N NaOH solution in the reactor, medium was then aseptically dispensed into a sterile 50-L carboy with medium outlets, medium filling, and sterile air inlet ports.

Glucose medium consisted of 100 g/L of glucose.

Sulfate medium was prepared as 10.8 g/L of Na_2SO_4, 0.46 g/L of $CaCl_2 \cdot 2H_2O$, 6.4 g/L of NH_4Cl, 3.2 g/L of $MgCl_2 \cdot 6H_2O$, and 3 g/L of KH_2PO_4.

Salt medium consisted of 0.46 g/L of $CaCl_2 \cdot 2H_2O$, 6.4 g/L of NH_4Cl, 3.2 g/L of $MgCl_2 \cdot 6H_2O$, and 3 g/L of KH_2PO_4.

Selenium medium had 14.4 g/L of Na_2SeO_3 or Na_2SeO_4, 0.46 g/L of $CaCl_2 \cdot 2H_2O$, 6.4 g/L of NH_4Cl, 3.2 g/L of $MgCl_2 \cdot 6H_2O$, and 3 g/L of KH_2PO_4.

Cane molasses medium consisted of 23 g/L cane molasses.

Nitrogenous medium contained one of the following complex nutrients at various concentrations as nitrogen and/or vitamin source: yeast extract, hydrolyzed beef serum concentrate (American Protein Co., Ames, IA), peptonized milk protein, soy peptone, gelatin peptone (Marcor, Hackensack, NJ), and cane molasses (Diamond V Mills, Cedar Rapid, IA). Each medium was prepared at 23 to 7.5 g/L depending on the desired concentration in 3-L carboys and sterilized in the autoclave for 1 h.

Yeast extract medium consisted of 7.5 g/L yeast extract and was prepared as described in the Nitrogenous Medium paragraph.

Medium Optimization. *Nitrogenous Nutrient.* Growth medium with no methionine or low methionine was desirable for selenium incorporation. Therefore, several nitrogenous sources were tested at various concentrations in a continuous reactor at 0.2 h^{-1} dilution rate. Glucose medium, sulfate medium, cane molasses medium, and nitrogenous medium were pumped into the reactor to produce the following medium blends in the reactor: 40 g/L of glucose, 4.5 g/L of Na_2SO_4, 0.19 g/L of $CaCl_2 \cdot 2H_2O$, 2.6 g/L of NH_4Cl, 1.3 g/L of $MgCl_2 \cdot 6H_2O$, 1.2 g/L of KH_2PO_4, 2 g/L of cane molasses, and a desired concentration of nitrogenous compound (0.1, 0.5, or 1.0 g/L). The reactor was operated until steady state (≥5 working volume changes) was established before changing to next medium composition.

Determination of Minimum Sulfate Concentration. The lowest possible concentration of sulfate with no deleterious effect on yeast growth was determined using a gradient delivery unit. The reactor was again operated at 0.2 h^{-1} by pumping glucose medium, cane molasses medium, yeast extract medium, salt medium, and sulfate medium with gradient. The dilution rate was again kept at 0.2 h^{-1}. The medium composition was the same but with 0.5 g/L yeast extract. A gradient delivery unit was developed that consisted of two vessels. The first vessel was a 10-L carboy containing salt medium. This was connected in series to a second vessel (mixing vessel), which was a 3-L flask with a stirring bar, containing 1.9 L of salt medium with sodium sulfate. The second vessel was sealed with a rubber stopper consisting of a medium inlet and medium outlet ports only. The medium outlet line was connected to the fermentor with a peristaltic pump. As salt medium was being pumped from the second vessel to the fermentor, the second vessel working volume was unchanged, whereas from the first vessel, medium was being added at the same rate as the pump flow rate. Therefore, the sodium sulfate in the second vessel was slowly being diluted as it was entering the fermentor. The amount of sodium sulfate leaving in the second vessel was calculated by the equation

$$C_s = C_i\, e^{-0.02151t/1.9} \quad (1)$$

where C_s is the sodium sulfate concentration at $t = t$ and C_i is the sodium sulfate concentration at $t = 0$ (10.8 g/L).

Sodium Selenite or Sodium Selenate Gradient. Two selenium sources were investigated: sodium selenite (Na_2SeO_3) and sodium selenate (Na_2SeO_4). The goal was to identify the optimal selenium-to-sulfur ratio in the reactor that would not have a negative effect on cell density. The reactor was operated at 0.2 h^{-1} by pumping glucose medium, salt medium, cane molasses medium, and yeast extract, and also selenium medium from the gradient delivery unit, producing a gradient of sodium selenite or sodium selenate. The medium in the mixing vessel contained 1.9 L of salt medium supplemented with 1.07 g/L Na_2SO_3, which produced 0.135 g/L Na_2SO_3 in the reactor as a minimum sulfur concentration. The first vessel contained sodium selenite or sodium selenate, whereas the mixing vessel contained neither at the beginning. Thus, the concentration gradient for selenium slowly increased in the mixing fermentor. The amount of sodium selenite or sodium selenate leaving the mixing vessel was calculated by the equation

$$C_s = C_i - C_i/(C_i\, e^{-0.02151t/1.9}) \quad (2)$$

where C_s is the concentration at $t = t$ and C_i is the concentration at $t = 0$.

Also, the fermentor was operated with a constant addition of sodium selenate to produce 0.51 g/L sodium selenate in the reactor. Effluent was collected and freeze-dried for atomic absorption analysis.

Analysis. Samples were analyzed for cell density by absorbance at 620 nm by using Spectronic 20 and for glucose concentration by Water's high-pressure liquid chromatograph with refractive index detector (Demirci and Pometto, 1997). Also, the samples containing selenium were examined by the methylene blue reduction time (MBRT) test (Nagodawithana et al., 1985). A 0.1 g dry sample equivalent of fermentation broth was measured by absorbance at 620 nm using a standard line equation. The broth was centrifuged at 3000*g* for 5 min. After the supernatant had been decanted, the pellet was washed with deionized water and recentrifuged. The washed pellet was resuspended in 7 mL of reducing solution, which was a 20% (w/v) solution of 1-thioglycerol in 0.20 N phosphate buffer at pH 5.5 in a screw-capped test tube. After shaking for 10 s, the solution was left standing for 3 min. The headspace of the test tube was purged with nitrogen, and a drop of 2% (w/v) methylene blue solution was added. After thorough shaking, the time required for the blue color to disappear was recorded. Higher reduction times correlated to higher concentrations of organically bound selenium.

Electrothermal atomic absorption spectroscopy (EAAS) at the ISU Analytical Services Laboratory was used to determine the concentration of selenium in yeast which was freeze-dried after several washings with deionized water to remove culture medium. A modified method of Robberecht et al. (1982) was used for sample preparation; 0.1 g of freeze-dried yeast sample was predigested in 10 mL of 70% (v/v) nitric acid overnight at room temperature. The solution was heated at 75 °C for 24 h and then at 120 °C for 6 h with a condenser. After the condenser was removed, solution was maintained at 120 °C until the volume was reduced to 4 mL. Then 5 mL of 70% perchloric acid was added and heated to 215 °C for 30 min with a condenser. The solution was cooled, 1 mL of concentrated HCl was added, and the solution was heated for 5 min at 100 °C to reduce selenium to the quadrivalent state. After digestion was completed, the solution was transferred to a 100-mL volumetric flask, 3.6 mL of 70% nitric acid was added, and the solution was brought to volume with ultrapure water with a specific resistivity of 18 MΩ/cm, which was obtained by a Milli-Q purifier (Millipore, Milford, MA). A 1-mL aliquot was transferred into a 25-mL volumetric flask and made up to

Table 1. Effect of Various Complex Nitrogenous Compounds at Various Concentrations on Cell Density

complex nitrogen compound	cane molasses (%)	biomass (g/L)
none	0.2	0.89
none	0.4	1.23
0.1% corn steep liquid[a]		2.82
0.1% corn steep liquid	0.2	2.69
0.05% corn steep liquid	0.2	2.02
0.01% corn steep liquid	0.2	1.23
0.1% soy peptone[a]		1.36
0.1% soy peptone	0.2	2.49
0.05% soy peptone	0.2	1.96
0.01% soy peptone	0.2	1.56
0.1% gelatin peptone[a]		0.03
0.1% gelatin peptone	0.2	1.86
0.05% gelatin peptone	0.2	1.76
0.01% gelatin peptone	0.2	1.46
0.1% hydr beef fibrin[b]	0.2	1.89
0.05% hydr beef fibrin	0.2	1.89
0.01% hydr beef fibrin	0.2	2.56
0.1% yeast extract[c]		3.50
0.1% yeast extract	0.2	4.32
0.05% yeast extract	0.2	4.07
0.01% yeast extract	0.2	1.83

[a] Supplied by Marcor, Hackensack, NJ. [b] Supplied by American Protein Co., Ames, IA. [c] Supplied by Champlain Industries, Inc., Clifton, NJ.

volume by 6.5% nitric acid containing 5.208 g/L nickel as a matrix modifier. Selenium in the resulting solution was measured by Thermo Jarrel Ash Smith Hieftje 12 AA/AE electrothermal atomic absorption spectrometry (EAAS) at 196 nm, with a spectral slit width of 2 nm, ashing temperature of 500 °C, and atomization temperature of 2300 °C.

RESULTS AND DISCUSSION

Medium Optimization. The desired culture medium would produce high cell yields in medium containing low concentrations of the sulfur-containing amino acids (methionine and cysteine). The continuous fermentation with nitrogenous compound concentrations of 1, 0.5, or 0.1 g/L was operated at a dilution rate of 0.2 h^{-1}. Also, fermentations were performed at 0.1 g/L of nitrogenous compound concentration without cane molasses in addition to 2 or 4 g/L of cane molasses without any nitrogenous compound. Cane molasses alone did not support yeast growth (Table 1). However, cane molasses with each nitrogenous nutrient stimulated yeast growth in almost every blend. For example, biomass was 0.03 g/L for 0.1% (w/v) gelatin peptone alone, whereas it was 1.86 g/L for medium containing 0.1% (w/v) gelatin peptone and 0.2% (w/v) cane molasses. Even yeast extract blend illustrated higher cell density when cane molasses was present in the medium. These results confirmed the requirement for the cane molasses supplement, a good source of vitamins and minerals, which was the ideal for our purpose.

In all cases, nitrogenous compounds at 1 g/L concentration had a significant effect on yeast growth. When all of the media were compared, corn steep liquor, soy peptone, gelatin peptone, and hydrolyzed beef fibrin demonstrated ~2−3 g/L biomass production, whereas yeast extract demonstrated >4 g/L biomass with cane molasses. According to the manufacturer, Ardamine-Z yeast extract has a low methionine content (0.74%).

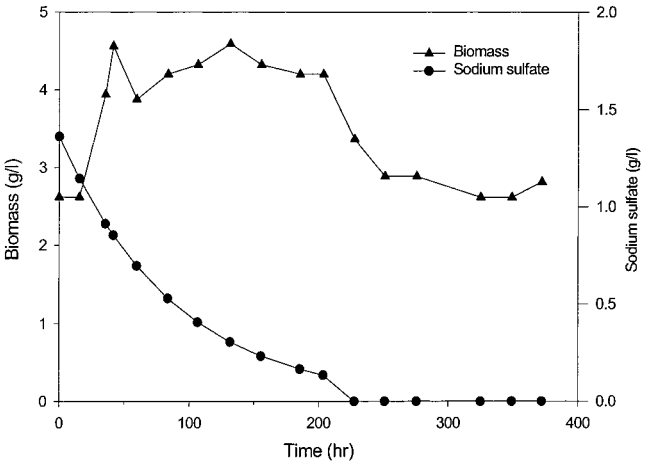

Figure 1. Effect of lowering sodium sulfate concentration with a gradient delivery system on cell density. Sodium sulfate concentration was calculated by using eq 1.

Furthermore, no significant difference in cell density was demonstrated between 1 and 0.5 g/L of yeast extract (6.65 and 6.27 g/L biomass, respectively). Therefore, 0.5 g/L yeast extract was selected to keep methionine content at a minimum concentration.

Determination of Minimum Sulfate Concentration. We believed that a low sulfur concentration in the medium was critical to stimulate incorporation of selenium into sulfur-containing amino acids such as methionine. To investigate the minimum amount of sulfur needed for healthy yeast growth, sodium sulfate concentration was gradually reduced from 1.360 to 0.135 g/L and then to zero (Figure 1). Cell density was stable at ~4 g/L biomass until 0.135 g/L sodium sulfate, and then it reduced to <3 g/L biomass when no sodium sulfate was added, which was the value before sulfate was introduced. This demonstrated that a minor concentration of sulfate was necessary for higher biomass. Therefore, 0.135 g/L sodium sulfate was selected for further fermentation, which was equivalent to 0.03 g/L sulfur.

MBRT. This test was used to evaluate the MBRT efficiency to determine the ratio of inorganic selenium to organically bound selenium in yeast biomass. When a solution of sodium selenite or sodium selenate was used, MBRT was in a matter of seconds. However, when a solution of selenomethionine (Sigma Chemical Co.) was used, MBRT was ~30 min. Therefore, a high MBRT indicated the presence of organically bound selenium. However, there was no linear relationship to the ratio of inorganic to organically bound selenium. Only when organically bound selenium was present was MBRT >20 min.

Sodium Selenite Gradient with Minimum Sulfate. To investigate the optimum selenium/sulfur (Se/S) ratio, the gradient delivery unit was utilized to increase the Se/S ratio from 0:1 to 10:1. MBRT sharply reduced immediately when sodium selenite was introduced, which indicated the presence of only inorganic selenium in the cell. Cell density decreased slowly until 1.1 g/L, especially after 0.3 g/L sodium selenite and a Se/S ratio of 4.6:1 (Figure 2), but then increased to ~1.7 g/L biomass at 0.6 g/L sodium selenite. These results suggest the development of a selenium-adapted strain in the fermentor. Therefore, culture sample (FD10) was aseptically recovered to preserve the adapted strain for further studies. Also, 1904 μg of selenium/g of dry

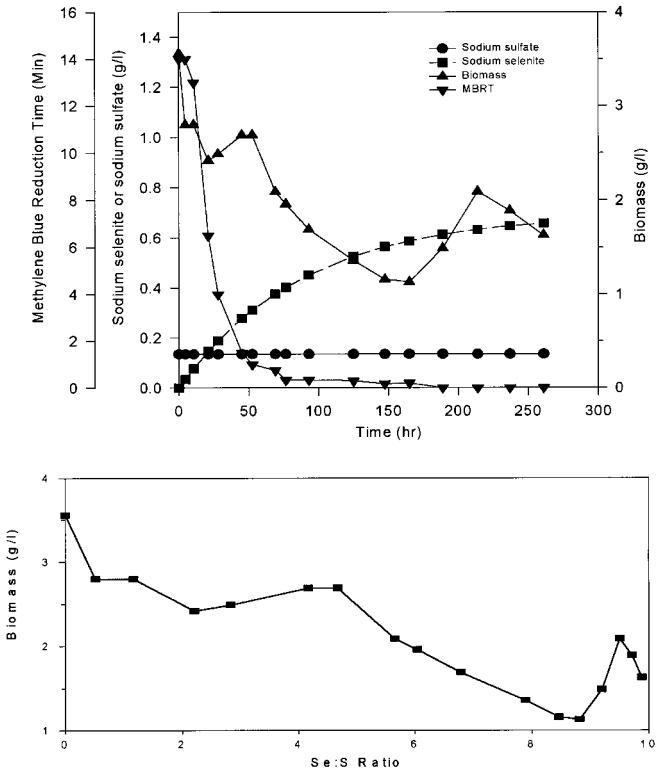

Figure 2. Continuous fermentation with increasing sodium selenite up to 0.65 g/L with a gradient delivery system in minimum sodium sulfate (0.135 g/L) medium. Sodium selenite concentration was calculated by using eq 2.

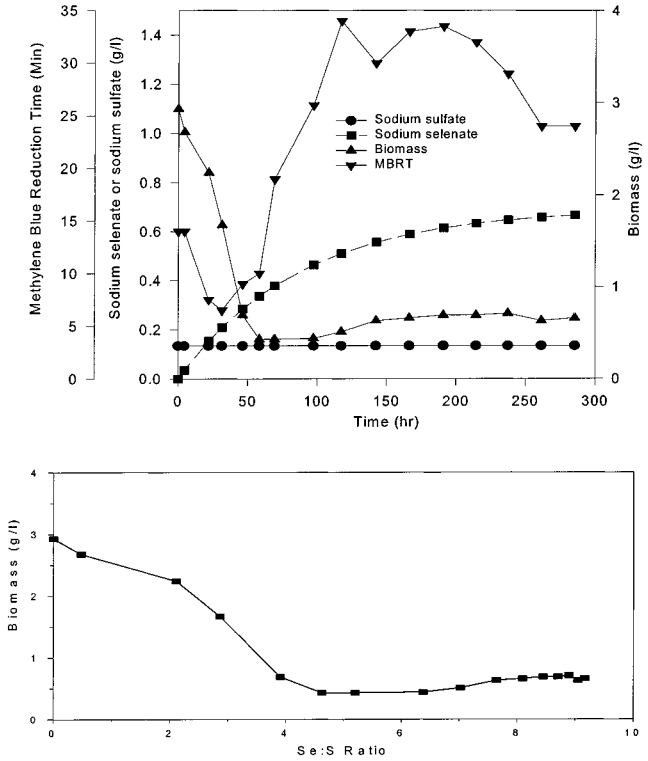

Figure 3. Continuous fermentation with increasing sodium selenate up to 0.69 g/L with a gradient delivery system in minimum sodium sulfate (0.135 g/L) medium. Sodium selenate concentration was calculated by using eq 2.

biomass was determined by atomic absorption analysis of freeze-dried yeast culture, which was harvested toward the end of the fermentation.

Sodium Selenate Gradient with Minimum Sulfate. Fermentation that targeted a Se/S ratio of 10:1 by gradient of sodium selenate demonstrated a sharp decrease just after the introduction of sodium selenate, whereas MBRT demonstrated a significant increase (34 min) after an initial decrease, which indicated production of organically bound selenium (Figure 3). However, MBRT started to decrease after it reached 34 min at 0.51 g/L sodium selenate, which was still significantly higher than that of sodium selenite. Atomic absorption analysis of freeze-dried culture collected toward the end of the fermentation demonstrated 248 μg of selenium/g of dry biomass. Culture sample (FD12) was aseptically recovered to preserve this adapted strain for further studies. Figure 3 shows that cell density decreased constantly until a Se/S ratio of 3.9:1. Therefore, fermentation was operated with the gradient system with Se/S ratio 0 to 3.9:1, which created a slower gradient permitting better selenium incorporation. Similarly, cell density dropped constantly to 0.7 g/L biomass but at a slower rate as sodium selenate concentration increased (Figure 4). Also, MBRT demonstrated a reduction followed by an increase ~25 min, which possibly indicates incorporation of selenium as yeast grows. More importantly, EAAS analysis confirmed high selenium incorporation into yeast (687 μg of selenium/g of dry biomass). Culture sample (FD18) was aseptically recovered to preserve this adapted strain for further studies.

Finally, fermentation was performed at a constant concentration of 0.51 g/L sodium selenate, which demonstrated the highest MBRT (34 min), and a Se/S ratio of 7.0. After steady state was reached, culture sample

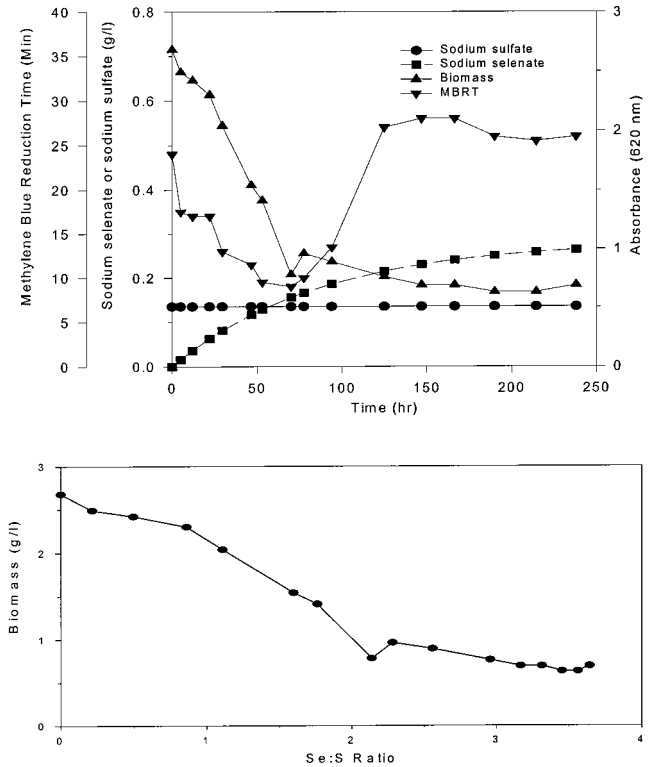

Figure 4. Continuous fermentation with increasing sodium selenate up to 0.28 g/L with a gradient delivery system in minimum sodium sulfate (0.135 g/L) medium. Sodium selenate concentration was calculated by using eq 2.

(FD14) was aseptically recovered to preserve this adapted strain for further studies. Final biomass concentration was 0.76 g/L, which was not significantly different from previous fermentations. Also, atomic absorption analysis

of freeze-dried yeast culture, which was harvested toward the end of the fermentation, demonstrated 382 μg of selenium/g of dry biomass with a 30 min MBRT.

Conclusion. Continuous fermentations in optimum medium with minimum sulfate (0.135 g/L of sodium sulfate), 0.5 g/L of yeast extract, and 0.65 g/L of sodium selenite (Na_2SeO_3) demonstrated higher biomass and selenium content compared to the fermentation with sodium selenate (Na_2SeO_4). However, the MBRT was extremely low, which indicated that most of the selenium was in the inorganic form. On the other hand, fermentations performed in the same optimum medium with various sodium selenate concentrations produced lower biomass and less selenium content compared to the fermentation with sodium selenite. However, the MBRT was longer, which indicated a higher concentration of organically bound selenium. Fermentation targeted a 0.28 g/L sodium selenate concentration (Se/S ratio of 3.9:1 and biomass/Se ratio of 5.5:1) and was optimal with 687 μg of selenium/g of dry biomass. For scale up, these ratios can be adapted to fed-batch fermentation for increased cell densities and increased production of organically bound selenium.

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