Enhanced Organically Bound Selenium Yeast Production by Fed-Batch Fermentation†

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This study describes a fed-batch fermentation protocol for enhanced production of *Saccharomyces cerevisiae* containing organically bound selenium. Two levels of sodium selenate concentrations were applied as either a single dose or continuous addition. Fermentations with high sodium selenate (63.2 g/L in cane molasses feeding medium) demonstrated 24 g/L of biomass with 1382 μg of selenium/g of dry biomass for single-dose addition and 40 g/L of biomass with 1491 μg of selenium/g of dry biomass for continuous addition. Low selenium concentration (31.6 g/L in cane molasses feeding medium) demonstrated higher biomass concentration with higher selenium level; 37 g/L of biomass with 2846 μg of selenium/g of dry biomass and 45 g/L of biomass with 2495 μg of selenium/g of dry biomass for single-dose and continuous addition, respectively. Also, two adapted *S. cerevisiae* strains were evaluated in fed-batch fermentation. A single dose of low concentration demonstrated >3000 μg of selenium/g of dry biomass, but biomass concentration was lower (≤32 g/L) for these adapted strains.

Keywords: *Sodium selenate; selenium; yeast; Saccharomyces cerevisiae; fermentation*

INTRODUCTION

Saccharomyces cerevisiae has been used in brewing and bread making for several thousand years. Each of these traditional processes converts substrates into ethanol, carbon dioxide, and biomass. Ethanol is used as renewable fuel, and yeast biomass is used as a source of micronutrients for animal and human diets. High biomass production yields are obtained under aerobic growth conditions and low sugar concentration (<2 g/L), whereas under high sugar concentration (>2 g/L), sugar is metabolized into ethanol and CO_2, which decreases biomass yield. This phenomenon of ethanol production under aerobic condition is known as "the Crabtree effect" (Aiba et al., 1976; Woehrer and Roehr, 1981). In ideal conditions, it was reported that 245 g of dry weight of yeast per kilogram of cane molasses containing 480 g of sucrose can be obtained, which represents ~50% yield (Rosen, 1987). Cane molasses provides not only substrate but also important vitamins and minerals required for yeast growth. However, yeast fermentation media containing cane molasses are typically supplemented with biotin, because can molasses has between 0.5 and 0.8 ppm of biotin (Reed and Nagodawithana, 1991).

S. cerevisiae has commonly been used as a nutritional supplement to animal feed. Organically bound selenium has been accepted as an essential element for the growth of animals and humans. It has been shown that selenium has a cancer-protective effect (Combs, 1997) and has a profound effect on survival in HIV-infected patients (Bologna et al., 1994). Some other effects are still under investigation. In animal and human diets organically bound selenium in yeast is preferred for better bioavailability. Korhola et al. (1986) reported development of yeast with 500 μg of selenium/g of dry biomass. Nagodawithana et al. (1985) demonstrated organically bound selenium yeast production with minimum 1000 μg of selenium/g of dry biomass by using sodium selenite salt as the selenium source. Also, Calomme et al. (1995) reported production of organically bound selenium at concentrations of 253 μg/g of biomass for *Lactobacillus delbrueckii*, 375 μg/g of biomass for *L. plantarum*, and 407 μg/g of biomass for *L. casei*. Previously, we have optimized the production of organically bound selenium by continuous fermentation from inorganic selenium (Na_2SeO_3 and Na_2SeO_4) (Demirci and Pometto, 1999). In this paper, we developed a fed-batch fermentation protocol for the production of organically bound selenium by using a wild strain of *S. cerevisiae* and two adapted strains obtained during our previous study (Demirci and Pometto, 1999).

MATERIALS AND METHODS

Microorganism. *S. cerevisiae* (ATCC 26787) and two adapted strains obtained from continuous fermentation with sodium selenite or sodium selenate addition, *S. cerevisiae* FD10 and *S. cerevisiae* FD14 (Demirci and Pometto, 1999), respectively, were maintained as a freeze-dried culture and as a working culture in broth stored at 4 °C. The culture medium used contained 20 g/L of glucose, 6 g of yeast extract (Ardamine-Z, Champlain Industries, Inc., Clifton, NJ), 0.23 g of $CaCl_2 \cdot 2H_2O$, 5.4 g of Na_2SO_4, 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. All fermentation inocula were prepared from 24-h static culture at 30 °C.

Media Preparation. Unless otherwise stated, medium was sterilized in a B.B. Braun U-100 reactor (Allentown, PA) with

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† Journal Paper J-18055 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA, Project 3253, supported by Hatch Act and State of Iowa funds.

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10.1021/jf9811976 CCC: $18.00 © 1999 American Chemical Society
Published on Web 05/25/1999

Table 1. Final Values in 5-L Fed-Batch Fermentation with Sodium Selenate with *S. cerevisiae* (Wild Type), *S. cerevisiae* (FD10), or *S. cerevisiae* (FD14)

culture	sodium selenate addition	dry biomass (g/L)	biomass yield (%)	ethanol (g/L)	selenium (μg/g of dry biomass)	MBRT (min)
wild	no sodium selenate (control)	55.04	51	0.41	<248	13.0
wild	LS[a] added as single dose	36.66	38	0.69	2846	7.0
wild	HS[b] added as single dose	24.40	26	7.35	1382	7.5
wild	LS added continuously	45.24	47	0.26	2495	6.5
wild	HS added continuously	39.72	39	2.80	1491	3.7
FD10	LS added as single dose	32.1	31	0.00	3231	11.5
FD14	LS added as single dose	22.6	22	4.80	3259	8.0
FD10	LS added continuously	41.3	45	0.18	1781	9.0
FD14	LS added continuously	43.4	47	0.00	1466	4.5
wild	100 L fed-batch, LS added continuously	45.9	50	0.00	2384	10.0

[a] LS, low sodium selenate (31.6 g/L of CMF). [b] HS, high sodium selenate (63.2 g/L of CMF).

constant agitation (220 rpm) at 121 °C for 30 min. A sterile KH_2PO_4 solution was added aseptically to media in the reactor before dispensing. A 4 N NaOH solution was used to adjust the pH to 5.5. The sterile medium was then aseptically dispensed into presterilized 50-L carboys with medium outlets, medium filling, and sterile air inlet ports.

Trace element solution was prepared as 3.0 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of $MnSO_4 \cdot H_2O$, 1.0 g of NaCl, 0.1 g of $FeSO_4 \cdot 7H_2O$, 0.18 g of $CoSO_4 \cdot 5H_2O$, 0.08 g of $CaCl_2 \cdot 2H_2O$, 0.1 g of $ZnSO_4 \cdot 7H_2O$, 0.01 g of $CuSO_4 \cdot 5H_2O$, 0.01 g of $Al_2(SO_4)_3 \cdot nH_2O$, 0.01 g of H_3BO_3, and 0.01 g of $Na_2MoO_4 \cdot 2H_2O$ per liter of deionized water.

Cane molasses feeding (CMF) medium consisted of 530 g/L cane molasses, 21.7 g/L urea, 0.14 g/L $CaCl_2 \cdot 2H_2O$, 4 g/L $NH_4H_2PO_4$, 0.94 g/L $MgCl_2 \cdot 6H_2O$, 3 g/L KH_2PO_4, and 1 mL/L trace element solution. Urea and KH_2PO_4 solution were filter sterilized and added to the medium aseptically after heat sterilization.

Cane molasses base (CMB) medium consisted of 7.5 mL/L cane feeding medium, 1.13 g/L Na_2SO_4, 0.14 g/L $CaCl_2 \cdot 2H_2O$, 0.94 g/L $MgCl_2 \cdot 6H_2O$, 3 g/L KH_2PO_4, 0.4 mg/L D-biotin, and 1 mL/L trace element solution.

An 80-mL sodium selenate solution was prepared by dissolving 24.6 or 49.3 g of sodium selenate in deionized water and autoclaving. For continuous sodium selenate addition experiments, sodium selenate was included in CMF. Resulting CMF medium had 31.6 g/L sodium selenate (CMF-LS) or 63.2 g/L sodium selenate (CMF−HS).

Fed-Batch Fermentation. A New Brunswick Bioflo 3000 benchtop reactor (Edison, NJ) equipped with pH, temperature, agitation, and anti-foam controls was employed. The 5-L working volume vessel was equipped with air-in and -out ports and alkali, acid, and medium inlet ports. Liquid breaks were placed on the medium feed lines into the reactor and the medium effluent line out of the reactor to prevent back flow of cells from the reactor into the medium feed and to prevent bioreactor contamination, respectively. Reactor medium pH was maintained at 5.5 with 4 N sodium hydroxide or 22 N phosphoric acid. Foaming was prevented by adding 1 mL of sterile Antifoam 289 (Sigma Chemical Co., St. Louis, MO) into the reactor before the fermentation started. Temperature was maintained at 30 °C. Aeration was maintained at 1 air volume/working volume per minute (vvm) (based on a final working volume of 4 L), and the agitation was 500 rpm. Fermentation started with 3 L of CMB medium, and then, depending on biomass concentration and doubling time, which determined the substrate utilization rate, fed by the feeding medium at a flow rate of 4 mL/h for flow rate 1 (F1) or 6 mL/h for flow rate 2 (F2) per liter. Throughout the fermentation, samples were taken and analyzed for cell density and ethanol concentration. At the end of fermentation, the reactor was drained, leaving enough inoculum for the next fermentation. The biomass in the drained medium was analyzed for selenium content. Purity of the culture in the reactor was periodically checked microscopicly throughout the fermentation.

For pilot-scale production, a B.B. Braun U-100 reactor was used with 45 L of starting base medium. All of the parameters were the same except that agitation was increased to 600 rpm. During the fermentation, periodically 0.5 L of sample was taken from the reactor aseptically for analysis. Selenium concentration was determined for cell-free medium and freeze-dried biomass.

Analysis. Samples were analyzed for cell density by absorbance at 620 nm by using Spectronic 20, and ethanol concentration was determined by a Water's high-pressure liquid chromatograph with refractive index detector (Demirci et al., 1997). To detect the presence of organically bound selenium, samples were examined by the methylene blue reduction time (MBRT) assay (Nagodawithana et al., 1985). For atomic absorption analysis, harvested medium was centrifuged, washed several times to remove solids from the medium, and freeze-dried. Samples were digested and evaluated using an electrothermal atomic absorption spectroscopy (EAAS) at the ISU Analytical Services Laboratory to determine the concentration of selenium in freeze-dried yeast (Demirci and Pometto, 1999).

RESULTS AND DISCUSSION

Medium Composition. The attempts to optimize medium composition for high biomass production demonstrated that use of cane molasses was crucial for yeast growth due to the presence of micronutrients. Therefore, the starting base medium in the reactor and feeding medium required cane molasses as the sole carbon source. To promote the growth further, 0.4 mg/L D-biotin was added to the starting base medium. Even though yeast extract produced ~10% more biomass (data not shown), urea was used as a nitrogen source to keep production costs low. Concentrated urea solution was filter-sterilized due to ammonia generation during heat sterilization.

The concentration of available sugar in the medium was very critical for biomass production. High carbohydrate concentration and dissolved oxygen initiated the Crabtree effect, which activates ethanol production (Aiba et al., 1976; Woehrer and Roehr, 1981). Therefore, sugar concentration was maintained at <2 g/L during fermentation, which was performed by continuous addition of CMF medium at different flow rates depending on the biomass concentration and yeast doubling time. The base medium was inoculated with a fresh yeast culture or with yeast from the previous fermentation. Our goal was to use all of the sugar in one doubling time, which maintained a negligible ethanol concentration (<1 g/L). The fermentation without any sodium selenate (control) demonstrated 55 g/L biomass with 51% yield in 98 h (Table 1). The ethanol concentration of 0.4 g/L indicated insignificant conversion of sugar into ethanol. MBRT was 13 min, and selenium was determined by EAAS as <246 μg/g of dry biomass.

Sodium Selenate Feeding. It was previously determined that the biomass/selenium ratio was 5.5:1 for

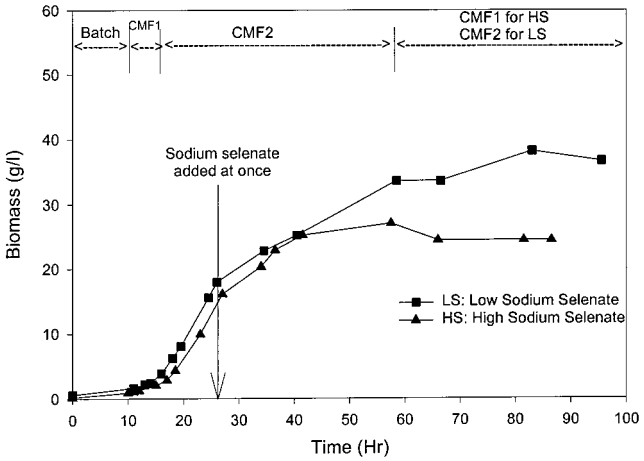

Figure 1. Five-liter fed-batch fermentation in cane molasses medium with a single dose of sodium selenate. CMF1 and CMF2 were cane molasses feeding medium at flow rates of 4 and 6 mL/h per liter of starting base medium, respectively.

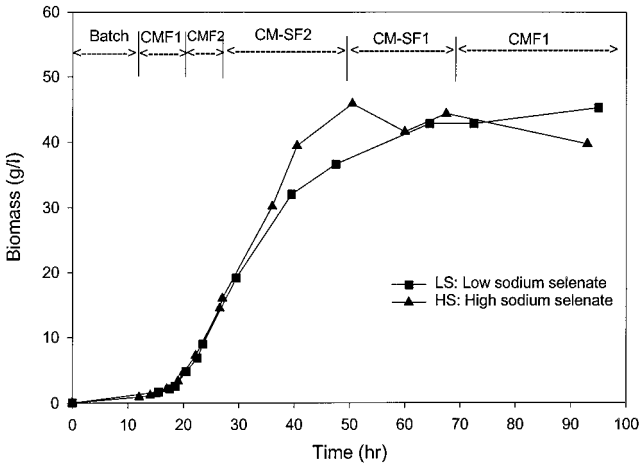

Figure 2. Five-liter fed-batch fermentation in cane molasses medium with continuous sodium selenate addition. CMF1 and CMF2 were cane molasses feeding medium at flow rates of 4 and 6 mL/h per liter of starting base medium, respectively. CM-SF1 and CM-SF2 were cane molasses−sodium selenate feeding medium at flow rates of 4 and 6 mL/h per liter of starting base medium, respectively.

high selenium incorporation in continuous fermentation. Therefore, two different levels of sodium selenate concentration were used. Low sodium selenate (LS) level was based on 20 g/L biomass concentration when sodium selenate addition was initiated. High sodium selenate (HS) level was based on a targeted 40 g/L biomass at the end of fermentation. Also, low- or high-level sodium selenate was added into the reactor either by a single dose of concentrated solution or by continuous addition with CMF medium. When the single dose of sodium selenate was added, fed-batch fermentation with LS demonstrated 36.6 g/L biomass and 0.69 g/L ethanol, whereas HS demonstrated 24.4 g/L biomass and 7.35 g/L ethanol (Figure 1). High ethanol production in HS resulted from a high free sugar concentration. LS not only produced higher biomass but also incorporated more selenium, 2846 μg of selenium/g of dry biomass compared to 1382 μg of selenium/g of dry biomass for HS (Table 1). In both cases, MBRTs were almost the same. To increase biomass production and to reduce the toxic effects of sodium selenate, sodium selenate was added continuously. Therefore, CMF with sodium selenate was used to feed the reactor starting

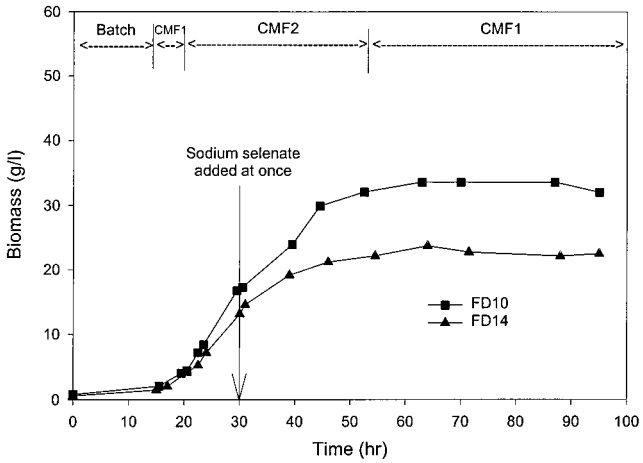

Figure 3. Fed-batch fermentation in cane molasses medium with a single dose of sodium selenate at low level with FD10 or FD14. CMF1 and CMF2 were cane molasses feeding medium at flow rates of 4 and 6 mL/h per liter of starting base medium, respectively.

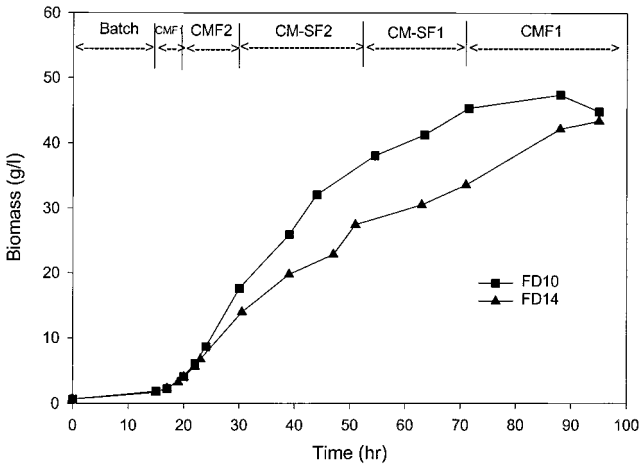

Figure 4. Five-liter fed-batch fermentation in cane molasses medium with continuous sodium selenate addition at low level with FD10 or FD14. CMF1 and CMF2 were cane molasses feeding medium at flow rates of 4 and 6 mL/h per liter of starting base medium, respectively. CM-SF1 and CM-SF2 were cane molasses−sodium selenate feeding medium at flow rates of 4 and 6 mL/h per liter of starting base medium, respectively.

before 20 g/L biomass was reached in the reactor. Final biomass concentrations were 45.2 and 39.7 g/L biomass for LS and HS, respectively (Figure 2). Again, the selenium content of dried yeast for LS (2495 μg/g of dry biomass) was higher than that of HS (1491 μg/g of dry biomass). Also, LS demonstrated higher MBRT as an indication of more organically bound selenium (Table 1).

Fed-batch repeated fermentations with adapted strains of *S. cerevisiae* FD10 and FD14 were performed for LS (Figure 3). When the single dose of sodium selenate was added, FD10 demonstrated higher biomass production and biomass yield than FD14 (Figure 3). Both FD10 and FD14 demonstrated similar selenium incorporation: 3231 and 3259 μg of selenium/g of dry biomass, respectively (Table 1). However, the MBRT for FD10 was higher than for FD14. When sodium selenate was added continuously before biomass concentration reached 20 g/L, biomass concentrations of FD10 and FD14 were higher, 41.3 and 43.4 g/L, respectively (Figure 4). Again, FD10 demonstrated higher selenium incorporation (1781 μg/g dry biomass) than that of FD14 (1466 μg/g of dry

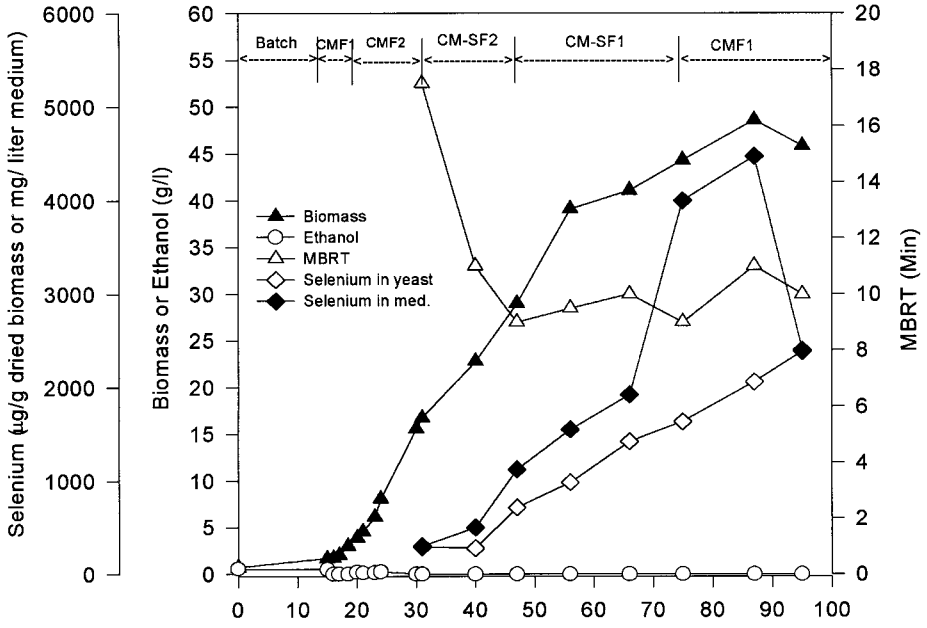

Figure 5. Hundred-liter fed-batch fermentation in cane molasses medium with 8.21 g/L continuous sodium selenate addition at low level in a 100-L Braun bioreactor. CMF1 and CMF2 were cane molasses feeding medium at flow rates of 6 and 4 mL/h per liter of starting base medium, respectively. CM-SF1 and CM-SF2 were cane molasses−sodium selenate feeding medium at flow rates of 4 and 6 mL/h per liter of starting base medium, respectively.

biomass). Similarly, selenium incorporation and MBRT were higher for FD10 (Table 1).

To make a comparison, atomic absorption analysis and an MBRT test were performed on two different commercially available selenium yeasts: Red Star Se yeast and Nutrex Se 1000 (Red Star of Universal Foods, Milwaukee, WI). Selenium concentration and MBRT were 1251 μg/g of dry biomass and 5.5 min, respectively, for Red Star Se yeast and 857 μg/g of dry biomass and 0.4 min, respectively, for Nutrex Se 1000. We assumed that Red Star Se yeast was produced by using the protocol suggested by Nagodawithana et al. (1985). They demonstrated minimum 1000 μg/g of dry biomass, which was also confirmed by our analysis. However, the organically bound selenium yeast produced in this study demonstrated ~2 times more selenium incorporation and higher MBRT, which indicated improved production of organically bound selenium.

Pilot-Scale Fed-Batch Fermentation. Fermentation with wild type *S. cerevisiae* with continuous addition of sodium selenium was performed in 100-L pilot-scale fermentation. A longer lag time was observed, which might be due to aeration and agitation differences affecting the dissolved oxygen level in the reactor. Final biomass concentration was 45.9 g/L (50% yield). Ethanol concentration was not detectible by HPLC, and MBRT was 10 min. This large-scale fermentation demonstrated similar results with bench-scale except MBRT, which was higher for pilot-scale fermentation (Table 1). EAAS analysis on dried biomass demonstrated selenium continuously increased to 2384 μg/g of dry biomass (Table 1; Figure 5). Selenium accumulated in the medium, which was needed to produce a positive pressure on cells for incorporation until selenium addition stopped, which decreased because of continued incorporation into yeast cells. As a result, pilot-scale fermentation demonstrated similar performance in terms of biomass and ethanol production, biomass yield, and incorporated selenium content except MBRT, which was higher for large-scale fermentation. This increase in MBRT indicates a higher concentration of organically bound selenium in the yeast biomass.

Overall, fermentations done in a 5-L benchtop fermenter and a 100-L pilot-scale fermenter demonstrated very similar biomass concentrations (~45 g/L) and selenium concentrations in dried yeast (~2400 μg/g of dry biomass). These results confirm that the process is reproducible.

Conclusion. Organically bound selenium yeast was produced in a fed-batch fermentation with continuous feeding of sodium selenate. Use of cane molasses was necessary to obtain high biomass production, but it must be added to the fermentor continuously to prevent a high concentration of free sugar (<2 g/L of sugar). Utilization of HS demonstrated lower biomass production and yield because of higher toxic effect on yeast cells. LS, which correlated to 20 g/L biomass, demonstrated the best results when it was added to the reactor continuously with cane molasses feeding medium. Adapted strains (FD10 and FD14) demonstrated the highest selenium incorporation, which was >3200 μg/g of dry biomass with 11.5 and 8 min MBRTs, respectively, when sodium selenate was added continuously at LS level. However, they demonstrated lower biomass production and yield. These results clearly demonstrate enhanced production of organically bound selenium yeast in fed-batch fermentation.

ACKNOWLEDGMENT

We thank the ISU Fermentation Facility for its equipment used for this research and ISU Analytical Services Laboratory for electrothermal atomic absorption spectroscopy analysis.

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Received for review November 3, 1998. Revised manuscript received March 31, 1999. Accepted April 21, 1999. This research was supported by Diamond V Mills, Cedar Rapids, IA; the Center for Advanced Technology Development at ISU; and the Iowa Agriculture and Home Economics Experiment Station.

JF9811976