Enhanced Organically Bound Chromium Yeast Production†

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This paper describes continuous and fed-batch fermentation protocols for enhanced production of organically bound chromium yeast. During continuous fermentation, several inorganic chromium compounds were evaluated. Sodium chromate demonstrated the best chromium incorporation into yeast biomass without any precipitation in the fermentation medium. During fed-batch fermentation, several sodium chromate concentrations were evaluated at 1.1, 3.5, 5.9, 7.1, 8.3, and 10.8 g/L with continuous or single-dose addition. For single-dose addition of sodium chromate, some precipitation was observed for all concentrations, which reduced the available chromium in the fermentation medium. Adapted strain C11-1, obtained during continuous fermentation, produced higher biomass than the wild type but significantly lowered chromium incorporation. Pilot-scale fermentation demonstrated similar total chromium incorporation (2966 ppm) with lower biomass production compared to benchtop fermentation performed at the same sodium chromate addition.

Keywords: *Sodium chromate; chromium; yeast; Saccharomyces cerevisiae; fermentation*

INTRODUCTION

Two elements known for their toxicity at high concentration are selenium and chromium. However, they have recently been recognized as essential dietary supplements. Inorganic selenium and chromium are generally less bioavailable and toxic at high concentrations compared with organically bound forms. Therefore, we recently have developed a fed-batch fermentation protocol for enhanced organically bound selenium yeast production (Demirci et al., 1999b). In this paper, we describe the protocol for enhanced organically bound chromium yeast production.

Chromium is the sixth most abundant element in the earth's crust, and it is used in stainless steels, leather tanning, chrome-plated metal, and pigments for paints and ink (Mowat, 1997). It occurs in oxidation states of 0, +2, +3, and +6. Hexavalent chromium has been shown to be toxic (Akutsa and Fairhall, 1934). Hexavalent chromium compounds are strong oxidizing agents that can easily penetrate living cells. Workers exposed to high levels of chromium have symptoms of skin cancer, lung cancer, and hepatitis (Cohen et al., 1993). On the other hand, trivalent chromium is the most stable form and is known to be less toxic, because it is poorly absorbed by living cells (McDowell, 1992). Chromium is now recognized as a trace element essential for both animal and human nutrition (Burrows, 1983). Biological activity of chromium was first suggested by Curran (1954). Also, Schwarz and Mertz (1959) identified chromium as the active component of glucose tolerance factor (GFT), which is a cofactor for insulin. Furthermore, chromium was shown to improve serum lipid profile (Riales and Albrink, 1981; Offenbacher et al., 1985). When Cortisol was reduced, supplemental organically bound chromium demonstrated reduced need for preventative and therapeutic antibiotics and reduced heat stress (Mowat, 1997).

Organically bound chromium has greater biological activity than inorganic chromium (Vinson and Hsiao, 1985). Therefore, analysis of total chromium is not the optimal indicator of biologically active chromium. Toepfer et al. (1973) developed an ethanol extraction method for the analysis of biologically active chromium. Meats and whole-grain products are some of the best sources of organically bound chromium (McDowell, 1992). Chromium-enriched yeasts have been used as organically bound chromium sources. Skogerson (1982) demonstrated organically bound chromium yeast production with ~1000 ppm of chromium in dry yeast by using chromium chloride hexahydrate as the chromium source. However, Skogerson (1982) performed no screening for proper chromium source and illustrated no optimization for chromium concentration in the fermentation medium. More importantly, there were no data presented to confirm the presence of organically bound chromium in their yeast.

In this paper we describe a continuous fermentation method for screening inorganic chromium compound for an optimum chromium incorporation into yeast and a fed-batch fermentation protocol for the enhanced production of organically bound chromium by using a wild strain of *Saccharomyces cerevisiae* and one adapted strain obtained during continuous fermentation.

MATERIALS AND METHODS

Microorganism. *S. cerevisiae* (ATCC 26787) and the adapted strain (*S. cerevisiae* C11-1) obtained from continuous fermentation with sodium chromate addition were maintained as a freeze-dried culture and as a working culture in broth stored at 4 °C. The culture medium contained 20 g/L of glucose, 6 g of yeast extract (Ardamine-Z, Champlain Industries, Inc.,

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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.

Stock Medium Preparation. Bulk media (100 L) were sterilized in a B.B. Braun U-100 reactor (Allentown, PA) with constant agitation (220 rpm) at 121 °C for 30 min. When needed, a sterile KH_2PO_4 solution was added aseptically to media in the reactor before dispensing. Sterile medium was then aseptically dispensed into sterile 50-L carboy with medium outlets, medium filling, and sterile air inlet ports.

(a) Medium Composition for Continuous Fermentation. Minimal growth medium was used as suggested by Demirci and Pometto (1999a) for the same strain which contained the following: trace element stock solution had 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 $Na_2MoO_4 \cdot 2H_2O$ in 1 L of deionized water.

Glucose medium consisted of 100 g/L of glucose (Cerulose, Staley, Decator, IL) with no pH adjustment.

Yeast extract-salt medium was prepared as 0.95 g/L of yeast extract (Ardamine-Z), 0.25 g/L of $CaCl_2 \cdot H_2O$, 7.23 g/L of NH_4Cl, 1.70 g/L of $MgCl_2 \cdot 6H_2O$, 1.60 g/L of KH_2PO_4, 0.72 mg/L of d-biotin, and 1.8 mL/L of trace element solution. D-Biotin solution was filter sterilized and then added to the medium aseptically after heat sterilization.

Sulfate medium had 1.07 g/L of $NaSO_4$.

Cane molasses consisted of 28.7 g/L of cane molasses (supplied by Diamond V Mills, Cedar Rapids, IA) and was sterilized in a 50-L Braun Biostat U bioreactor and transferred into presterilized 10-L carboys.

Chromium medium was prepared with 1.07 g/L of $NaSO_4$ and one of the following chromium compounds (Aldrich Chemical Co., Inc., Milwaukee, WI): 2.29 or 0.69 g/L of chromium(VI) oxide; 6.09 g/L of chromium(III) chloride hexahydrate; 9.14 or 1 g/L of chromium(III) nitrate nanohydrate; 13.78 g/L of chromium(III) acetate hydroxide; 3.78, 0.75, 0.38, 0.15, 0.11, or 0.074 g/L of sodium chromate. Three liters of chromium medium was prepared in a 5-L carboy and autoclaved for 1 h.

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

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

Cane molasses−sodium chromate feeding (CMCF) medium with various amounts of sodium chromate (between 3.3 and 32.3 g) was dissolved in 80 mL of deionized water, autoclaved for 1 h, and then made up to 780 mL by adding sterile CMF medium. For single-dose additions, various amounts of sodium chromate were dissolved in 30 mL of deionized water and autoclaved. For large-scale fermentation, 322 g of sodium chromate was dissolved in 550 mL of deionized water and autoclaved, and then the volume was made up to 10 L by adding sterile CMF medium.

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 was equipped with air in- and out-ports, alkali and medium addition ports, and effluent side ports. Liquid breaks were placed on the medium feed lines into the reactor and the medium effluent line out of the reactor to prevent culture back flow from the fermentor into the medium feed and to prevent bioreactor contamination, respectively. Fermentor pH was maintained at 5.5 with 4 N NaOH for all the chromium compounds except chromium(III) acetate hydroxide, which was maintained at pH 4.0 to prevent precipitation. Temperature was maintained at 30 °C, and the fermentor working volume was 850 mL. Aeration was maintained at 1 working volume/air volume per minute (vvm).

The reactor was operated at 0.2 h^{-1} dilution rate by pumping 42.5 mL/h of glucose medium, 12 mL/h of cane molasses, 94 mL/h of yeast extract medium, and 21.5 mL/h of sulfate medium or chromium medium. The following composition was maintained in the feed stream throughout the fermentation: 25.0 g/L of glucose, 0.53 g/L of yeast extract, 2.02 g/L of cane molasses, 0.135 g/L of Na_2SO_4, 0.14 g/L of $CaCl_2 \cdot 2H_2O$, 4.0 g/L of $NH_4H_2PO_4$, 0.94 g/L of $MgCl_2 \cdot 6H_2O$, 0.89 g/L of KH_2PO_4, and 0−14 g/L of one chromium compound delivered by a gradient system. The mixing vessel contained 1.9 L of 1.07 g/L of Na_2SO_3, which produced 0.135 g/L of Na_2SO_3 in the reactor as a minimum sulfur concentration (Demirci and Pometto, 1999a). The first vessel contained chromium solution, whereas the mixing vessel contained neither at the beginning. Thus, the concentration gradient for chromium slowly increased in the mixing vessel. The concentration of the chromium compound leaving the mixing vessel was calculated by using the equation

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

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

Samples were drawn regularly for cell density and glucose analysis. Also, effluent was collected, centrifuged, and freeze-dried for chromium analysis.

Fed-Batch Reactor Design. A New Brunswick Bioflo 3000 benchtop reactor (Edison, NJ) equipped with pH, temperature, agitation with one paddle and two marine agitation blades, and antifoam controls was employed. The 5-L 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 culture flow 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 vvm (based on a final working volume of 4 L), and the agitation was 500 rpm. Fermentation started with 3 L of CMB medium then fed by the CMF or CMCF 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. Medium flow rates depended on the substrate utilization rate, which was determined by the biomass concentration and culture doubling time. Initial sucrose concentration in base medium was ∼2 g/L. During fermentation sucrose concentration was <2 g/L. 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 biomass to inoculate the next fermentation. The biomass in the drained medium was analyzed for chromium content. Culture purity in the reactor was periodically checked microscopically throughout the fermentation.

For pilot-scale fed-batch fermentations a B.B. Braun Biostat U reactor with three paddle agitation blades was used with 40 L of starting base medium. All parameters were the same as 5-L fermentations except agitation was 300 rpm.

Analysis. Samples were analyzed for optical cell density by absorbance at 620 nm by using a Spectronic 20 spectrophotometer (Milton Roy, Rochester, NY), which was then converted into dry biomass concentration by using a standard curve equation [biomass (g/L) = 0.666 × absorbance at 620 nm − 0.106]. Ethanol concentration was determined by a Waters high-pressure liquid chromatograph (Millipore Corp., Milford, MA) equipped with a Waters model 401 refractive index detector, column heater, autosampler, and computer controller (Demirci et al., 1997). Sucrose was analyzed by using

Table 1. Continuous Fermentation at 0.2 h^{-1} with Various Chromium Compounds

compound	precipitation in the fermentor	targeted sodium chromate concn (g/L)	targeted Cr/S ratio	chromium addition	biomass (g/L)	Cr concn in dry yeast (ppm)
chromium(VI) oxide	no	0.68	5:1	gradient	0.04	ND[a]
		0.087	1.5:1	gradient	0.15	
chromium(III) chloride hexahydrate	yes	0.77	5:1	gradient	2.90	ND
chromium(III) nitrate nanohydrate	yes	0.53	5:1	gradient	2.65	ND
chromium(III) acetate hydroxide[b]	no	0.87	2.5:1	constant	5.22	118
		1.74	5:1	constant	6.02	302
		3.47	10:1	constant	5.15	295
sodium chromate	no	0.177	5:1	gradient	0.10	ND
		0.035	1:1	gradient	0.10	ND
		0.047	0.5:1	gradient	0.10	ND
		0.019	0.2:1	constant	0.15	ND
		0.014	0.15:1	constant	0.55	555
		0.009	0.1:1:1	constant	2.20	373

[a] Not determined because of precipitation in the fermentor. [b] Stock medium was filter sterilized, and fermentor was operated at pH 4.0.

a YSI Select 2700 analyzer (Yellow Springs Instruments, Yellow Springs, OH).

For chromium analysis, harvested medium was centrifuged, washed several times with deionized water to remove solids from the medium, and freeze-dried. To determine total chromium analysis, 0.5 g of dry yeast was suspended in 10 mL of 70% HNO_3 and 4 mL of 37% HCl and then digested in a microwave with a nominal 630-W system (CEM Corp., Matthews, NC) at 140 °C for 25 min (10 min ramp and 15 min holding time). After the digests were diluted to 50 mL with 2% HNO_3, the solution was analyzed by an atomic absorption spectrometer (Perkin-Elmer Corp., Norwalk, CT) equipped with a flame detector at 357.9 nm and 0.7 slit width.

To determine the concentration of organically bound chromium in yeast, ethanol extraction was performed (Toepfer et al., 1973). A 0.1 g sample of freeze-dried yeast was resuspended in 30 mL of 50% ethanol in a 50-mL polycarbonate centrifuge tube. The mixture was heated for 5 min at 80 °C in an electrical autoclave. After cooling, tubes were centrifuged at 5000*g* for 5 min. The supernatant was filtered through a 0.45-μm Acrodisc HT Tuffryn membrane filter (Gelman Sciences, Ann Arbor, MI). After the pellet was resuspended in 15 mL of 50% ethanol, the tubes were centrifuged at 5000*g* for 5 min, and the supernatant was filtered as above. The filtered solution was placed in the 50-mL round-bottom flask, and the solution was dried using a rotary vacuum evaporator at 60 °C. Finally, the residue in the flask was dissolved in 50 mL of deionized water and analyzed for chromium by atomic absorption spectrometry as described above.

RESULTS AND DISCUSSION

Continuous Fermentation. Continuous fermentation at 0.2 h^{-1} was operated with five different chromium compounds with gradient or constant addition until steady state was achieved (Table 1). Chromium(VI) oxide was very toxic on yeast cells when the Cr/S ratio in the medium prior to growth was targeted to 5:1 and 1.5:1 with the gradient delivery system. Chromium(III) compounds were then evaluated because they are known to be less toxic (Mowat, 1997). Chromium(III) chloride hexahydrate and chromium(III) nitrate nanohydrate demonstrated 2.90 and 2.65 g/L biomass production, respectively. However, blue precipitate occurred in the fermentation medium. This not only decreased the amount of available chromium in solution but also remained within the pellet upon yeast recovery by centrifugation. Skogerson (1982), however, did not report any precipitation due to utilization of chromium(III) chloride hexahydrate.

Another chromium compound evaluated was chromium(III) acetate hydroxide. Because the stock medium of chromium(III) acetate hydroxide had precipitation after autoclaving, it was filter sterilized to avoid heat treatment. Continuous fermentation was performed at Cr/S ratios of 10:1, 5:1, or 2.5:1 at constant addition, not by gradient addition, because chromium(III) acetate hydroxide was not toxic to yeast cells. The ratios 10:1 and 5:1 demonstrated high biomass production (5.15 and 6.02 g/L, respectively) with ~300 ppm of chromium in dry yeast (Table 1). However, filter sterilization of chromium(III) acetate hydroxide stock solution and requirement of high concentration in fermentation medium would not be economical for large-scale fermentation.

Therefore, sodium chromate was evaluated for chromium incorporation into yeast cells. Continuous fermentation at 0.2 h^{-1} operated at Cr/S ratios of 5:1, 1:1, and 0.5:1 with gradient addition and 0.2:1, 0.15:1, and 0.1:1 with constant addition (Table 1). Targeted Cr/S ratios for 5:1, 1:1, and 0.5:1 were very toxic on yeast even though sodium chromate concentration was increased gradually with the gradient delivery system. Also, a Cr/S ratio of 0.15:1 was toxic, which demonstrated <0.1 g/L biomass production. However, constant addition of sodium chromate for Cr/S ratios of 0.15:1 and 0.1:1 demonstrated 0.26 and 1.36 g/L biomass production, respectively. Also, the chromium concentration in freeze-dried yeast was 555 ppm for a Cr/S ratio of 0.15:1, which was higher than that for a Cr/S ratio of 0.1:1 (373 ppm). This indicated that a minimum sodium chromate concentration in the medium for incorporation was 373 ppm. Also, culture samples recovered during fermentations for a Cr/S ratio of 0.15:1 were aseptically preserved as the possible adapted strain (C11-1) for further studies.

Fed-Batch Fermentation. Continuous fermentation gave us criteria to determine minimum and maximum concentrations that would be employed for fed-batch fermentation. Continuous fermentations with Cr/S ratios of 0.1:1 and 0.15:1 demonstrated sodium chromate/biomass ratios of 0.006 and 0.054, respectively. Therefore, the required sodium chromate concentration was calculated as 0.113 and 1.076 g/L sodium chromate for 20 g/L biomass concentration at which sodium chromate was introduced. However, the medium used for fed-batch fermentation with CBM had a higher sulfur

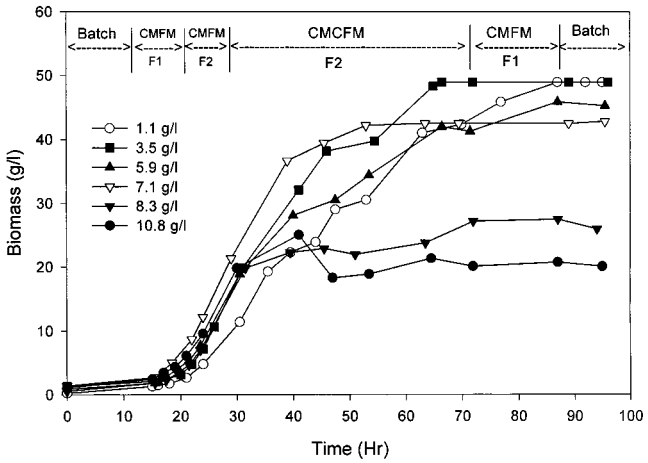

Figure 1. Fed-batch fermentation at various sodium chromate concentrations with continuous addition. CMFM, cane molasses feeding medium; CMCFM, cane molasses−sodium chromate feeding medium; F1 and F2, flow rates of 4 and 6 mL h^{-1} L^{-1} of starting base medium, respectively.

content than the minimal medium used for continuous fermentation. Therefore, these calculated sodium chromate concentrations were increased 10-fold to give 1.11 and 10.76 g/L sodium chromate as low and high end, respectively. The following concentrations also were evaluated: 3.5, 5.9, 7.1, and 8.3 g/L with continuous or single-dose addition.

Continuous Addition of Sodium Chromate. Fermentation was started as batch. When biomass concentration was doubled and sucrose concentration was ~0.5 g/L, continuous addition of CMF medium started at 4 mL h^{-1} L^{-1} and then 6 mL h^{-1} L^{-1} as biomass concentration increased. When biomass concentration was ~20 g/L, CMF medium was replaced by CMCF medium with various concentrations of sodium chromate. After all of the CMCF medium was added, the fermentation was continued by continuous addition of CMF medium at slow flow rate (4 mL h^{-1} L^{-1}). Finally, the fermentor was operated without feeding as batch toward the end of fermentation (Figure 1). Biomass production was similar up to 20 g/L. After sodium chromate was introduced, biomass production was effected depending on the concentration of sodium chromate. As sodium chromate concentration was increased from 1.1 to 10.8 g/L, final biomass concentrations decreased from 48.9 to 20.1 g/L (Figure 1). However, there were insignificant differences in biomass concentrations for fermentations with 1.1, 3.5, 5.9, and 7.1 g/L sodium chromate. Biomass concentration decreased dramatically for fermentations with 8.3 and 10.8 g/L sodium chromate. On the other hand, total chromium concentrations in the dry yeast were ~500 ppm up to 5.9 g/L sodium chromate, whereas it significantly increased to 3113, 4374, and 3766 ppm of chromium for 7.1, 8.3, and 10.8 g/L sodium chromate, respectively (Figure 2).

Toepfer et al. (1973) compared the biological activity of chromium and chromium analysis after ethanol extraction as an indirect measurement of biologically active chromium content. They reported a significant relationship between biological activity and chromium in ethanol extracts, which demonstrated that the amount of organically bound chromium could be estimated. Chromium analysis in ethanol extract followed the same pattern as in total chromium: organically bound chromium increased to 795, 1689, and 1745 ppm of dry yeast

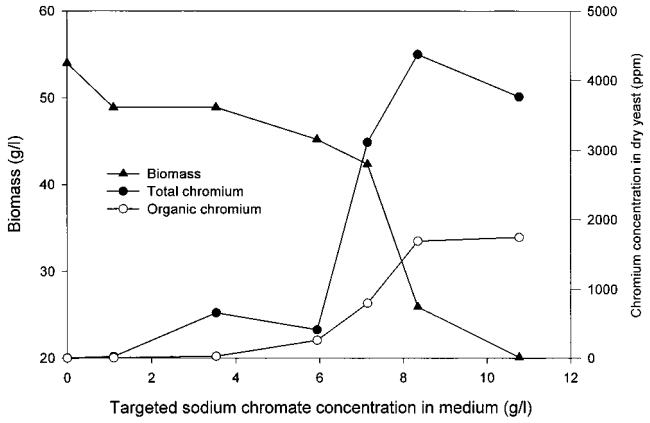

Figure 2. Relationship of biomass and organically bound chromium concentration in dry yeast with targeted sodium chromate concentrations in fed-batch fermentation medium.

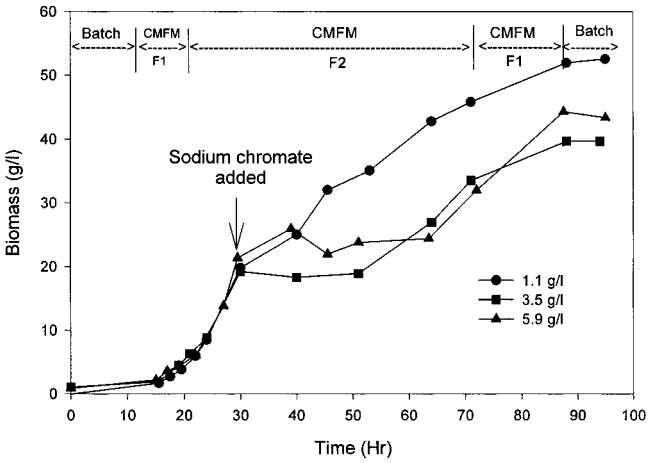

Figure 3. Fed-batch fermentation at various sodium chromate concentrations with single-dose addition. CMFM, cane molasses feeding medium; F1 and F2, flow rates of 4 and 6 mL h^{-1} L^{-1} of starting base medium, respectively.

as sodium chromate increased to 7.1, 8.3, and 10.8 g/L, respectively (Figure 2).

Single-Dose Addition of Sodium Chromate. To observe the effect of sodium chromate spiking, three concentration levels were selected, 1.1, 3.5, and 5.9 g/L sodium chromate. Sodium chromate solution was added at 20 g/L biomass (Figure 3). The fed-batch fermentation was continued by continuous addition of CMF medium as described previously. Results demonstrated that single-dose addition of sodium chromate demonstrated similar biomass production. However, single-dose addition did not benefit the chromium incorporation into yeast at 1.1 and 3.5 g/L sodium chromate concentrations, which were 187 and 597 ppm, respectively. However, 5.9 g/L sodium chromate concentration benefitted chromium incorporation to 1172 ppm, whereas it was 410 ppm for continuous addition. On the other hand, chromium analysis in ethanol extracts demonstrated 79, 269, and 343 ppm for 1.1, 3.5, and 5.9 g/L sodium chromate, respectively, and some precipitation was observed for all levels, which might have decreased the bioavailable sodium chromate. Therefore, single-dose addition was not a preferred method for organically chromium production.

Fed-Batch Fermentation with Adapted Yeast Strain (C11-1). Fermentations with *S. cerevisiae* adapted strain C11-1 at two sodium chromate concentrations of

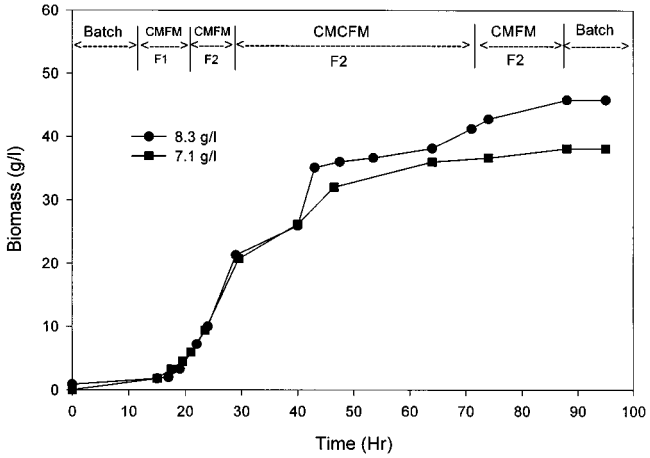

Figure 4. Fed-batch fermentation with *S. cerevisiae* adapted strain C11-1 at various sodium chromate concentrations with continuous addition. CMFM, cane molasses feeding medium; CMCFM, cane molasses−sodium chromate feeding medium; F1 and F2, flow rates of 4 and 6 mL h^{-1} L^{-1} of starting base medium, respectively.

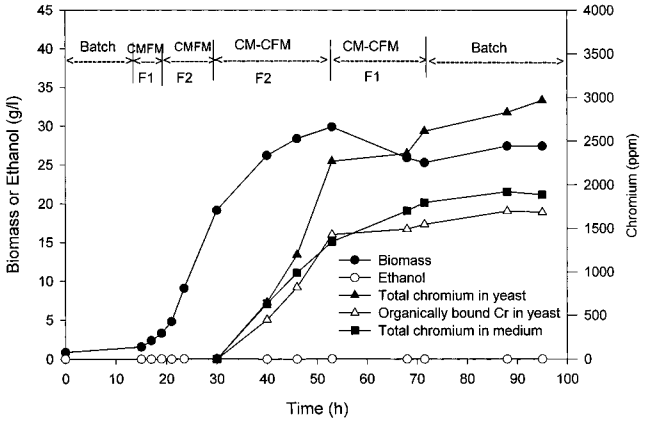

Figure 5. Pilot-scale fed-batch fermentation for targeted 7.1 g/L sodium chromate concentration based on the initial working volume with continuous addition. CMFM, cane molasses feeding medium; CMCFM, cane molasses−sodium chromate feeding medium; F1 and F2, flow rates of 4 and 6 mL h^{-1} L^{-1} of starting base medium, respectively.

7.1 and 8.3 g/L were performed (Figure 4). At 7.1 g/L sodium chromate, adapted strain C11-1 produced slightly higher biomass (46 g/L) compared with wild type at the same condition (42 g/L). At 8.3 g/L sodium chromate, adapted strain (C11-1) produced significantly higher biomass than wild type (38 and 26 g/L biomass, respectively). On the other hand, total chromium and organically bound chromium for adapted strain C11-1 were significantly lower: 95 and 4 ppm, respectively, at 7.1 g/L sodium chromate concentration and 1140 and 307 ppm, respectively, at 8.3 g/L sodium chromate concentration.

Pilot-Scale Fed-Batch Fermentation. On the basis of benchtop fermentations, fermentation with continuous addition of sodium chromate at high levels demonstrated ~4000 ppm of total chromium with low biomass production. On the other hand, 7.1 g/L sodium chromate demonstrated 3113 ppm of chromium incorporation and >40 g/L biomass production. Therefore, 7.1 g/L sodium chromate was selected as optimum chromium incorporation into yeast. Pilot-scale fed-batch fermentation targeted for 7.1 g/L sodium chromate concentration based on starting medium working volume (40 L) had a similar biomass production compared with benchtop fermentation until the time of CMCF medium addition at 30 h (Figure 5). For pilot-scale fermentation, final biomass concentration was <30 g/L, whereas for benchtop fermentation, final biomass concentration was ~40 g/L. This drop in biomass production most likely was due to a higher chromium concentration in the culture broth than desired. At each sampling time, 500 mL of fermentation medium was drawn from the reactor, which significantly reduced the working volume. Chromium addition was not corrected for these volume changes. Thus, the final chromium concentration was >5.5 g/L, which resulted in reducing biomass production.

At the end of the fermentation there was no ethanol production detected by HPLC, which indicated optimal feeding of the yeast fermentation for maximal biomass production. Also, total chromium in freeze-dried yeast was 2966 ppm, which is similar to that of benchtop fermentation. Organically bound chromium in freeze-dried yeast significantly increased to 1685 ppm. Thus, over half of the total chromium in the yeast biomass was organically bound. In the culture medium inorganic chromium concentration reached ~2000 ppm, which might be required for maximal chromium incorporation into yeast biomass. Overall, we believe that high biomass concentration with high chromium incorporation will be produced if final sodium chromate concentration is not >5.5 g/L. Also, a special precaution should be given the freshness of CMCF medium, because minimal chromium incorporation was achieved with 2-month-old CMCF (data not shown).

Conclusions. Among all of the evaluated chromium compounds, sodium chromate was best for the production of organically bound chromium in *S. cerevisiae*. Continuous fermentation demonstrated that Cr/S ratios of 0.1:1 and 0.15:1 were least toxic and produced high levels of chromium incorporation into yeast cells. For the fed-batch fermentation, continuous addition of sodium chromate medium for the targeted final concentration of 7.1 g/L based on the initial working volume was optimum for high chromium incorporation and biomass production. Chromium analysis of ethanol extract was used to estimate the concentration of organically bound chromium. Adapted strain C11-1 demonstrated higher biomass production but low production of organically bound chromium. Pilot-scale fermentation demonstrated similar total chromium incorporation with lower biomass production compared with benchtop fermentation performed at the same sodium chromate addition.

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