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High cell density fermentation of *Saccharomyces cerevisiae* GS2 for selenium-enriched yeast production

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Abstract−This paper describes a fed-batch fermentation protocol about production of selenium-enriched yeast. *Saccharomyces cerevisiae* GS2 was selected because of its high tolerance to selenium. The strain GS2 was tested and 122±0.5 g·l^{-1} dry cell weight was obtained after 30 h cultivation through feed back control of feed rate of glucose according to the concentration of ethanol and dissolved oxygen. Furthermore, the optimal pattern of Na_2SeO_3 addition was 9 mg Na_2SeO_3 against 1 g DCW at late exponential phase. With the combination of glucose feeding and Na_2SeO_3 addition, the final dry cell biomass reached 102±0.4 g·l^{-1} and a Se uptake level of 2,020±13 mg·Kg^{-1} was achieved in a 5 *l* fermentor after 38 h cultivation.

Key words: High Cell Density Fermentation, *Saccharomyces cerevisiae*, Selenium, Yeast

INTRODUCTION

The trace element selenium (Se) has been well recognized as an essential micronutrient for human and animals. At supranutritional dietary levels, Se can be used to prevent the development of many types of cancer [1-4] and have a profound effect on survival of HIV infected patients [5]. At higher concentrations, Se compounds can be either cytotoxic or possibly carcinogenic. Accordingly, sodium selenite, an inorganic Se compound, was reported to induce DNA damage, particularly DNA strand breaks and base damage [6].

It is generally believed that the ingestion of the organic Se compounds is better and safer than that of the inorganic Se. Some microorganisms produce biomass with high protein content and meanwhile transform inorganic Se (a low bioavailability, potentially toxic) into organic form (safe and highly bioactive). Hence, the safe use of selenium yeast is permitted as a source of selenium in animal feedstock for beef and dairy cattle by the Food and Drug Administration (www.epa.gov/fedrgstr/EPA-IMPACT/2002/July/Day-17/i17959.htm).

Previous work has shown that yeast is a good carrier for Se biotransformation. Suhajda et al. found that the addition of 30 μg·ml^{-1} sodium selenite to the batch cultures during the exponential growth phase resulted in 1,200-1,400 μg·g^{-1} Se content in the baker's yeast [7]. Demirci et al. [8,9] described fed batch and continuous fermentation protocols for incorporation of the selenium oxyanions, selenite and selenate, in *S. cerevisiae*. They found that a single dose of a low selenium concentration resulted in >3,000 μg·g^{-1} Se content, but the biomass concentration was lower (≤32 g·l^{-1}) after 95 h cultivation. The kinetics of selenite uptake by *S. cerevisiae* was characterized by Gharieb et al. [10]. However, low productivity of Se yeast is a major limitation to common commercialization.

Our laboratory has studied feeding control strategy to efficiently obtain high cell density about yeast fermentation [11,12]. The primary object in this work is to construct a simple and robust control strategy for Se-enriched yeast production. We describe a fed-batch fermentation protocol for Se-enriched yeast by direct glucose feedback control and single dose addition of sodium selenite.

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MATERIALS AND METHODS

1. Microorganism

87 yeast strains from different genera and species were tested to identify a strain with high biomass and high enrichment of Se. Cells of strains for selection were suspended respectively in 1 ml sterilized saline for at 25 °C for 4 h. Each suspension of starved cells were inoculated on YEPD plates (yeast extract, 10 g·l^{-1}; peptone, 20 g·l^{-1}; glucose, 20 g·l^{-1}; agar, 20 g·l^{-1}) containing six Se concentrations: 200, 300, 400, 500, 600, 700 mg·l^{-1} sodium selenite. GS2 showed the highest tolerance to Se (600 mg·l^{-1} sodium selenite) as well as Se accumulation potential after repeating the procedure 3 times. Analysis of the rDNA sequence of the D1/D2 domain of 20S rDNA gene from strain GS2 revealed that it was closely related to *S. cerevisiae* CBS 1171^T (Fig. 1).

2. Medium

YEPD medium containing 10 g·l^{-1} yeast extract, 20 g·l^{-1} peptone, 20 g·l^{-1} glucose.

Fermentation medium consisted of 60 g·l^{-1} glucose, 15 g·l^{-1} yeast extract, 8 g·l^{-1} $(NH_4)_2HPO_4$, 5 g·l^{-1} $MgSO_4$, 28 g·l^{-1} sugar cane molasses, 16 g·l^{-1} corn steep liquor, 1 g·l^{-1} K_2HPO_4, 1 g·l^{-1} KH_2PO_4, 10 mg·l^{-1} $ZnSO_4$, 6 mg·l^{-1} $FeSO_4$, 6 mg·l^{-1} $CuSO_4$, 6 mg·l^{-1} $MnSO_4$.

Feeding medium was 600 g·l^{-1} glucose.

Selenium medium was 20 g·l^{-1} sodium selenite.

3. Fermentor

The main equipment used in our experiments included a 5 *l* fermentor with temperature controller (Shanghai Baoxin Co., Ltd., Shanghai, China), pH and dissolved oxygen (DO) controller (Mettler - Toledo Instruments Co., Ltd., Switzerland). Ethanol concentration was measured on-line by a tubing sensor (FC-2002, East

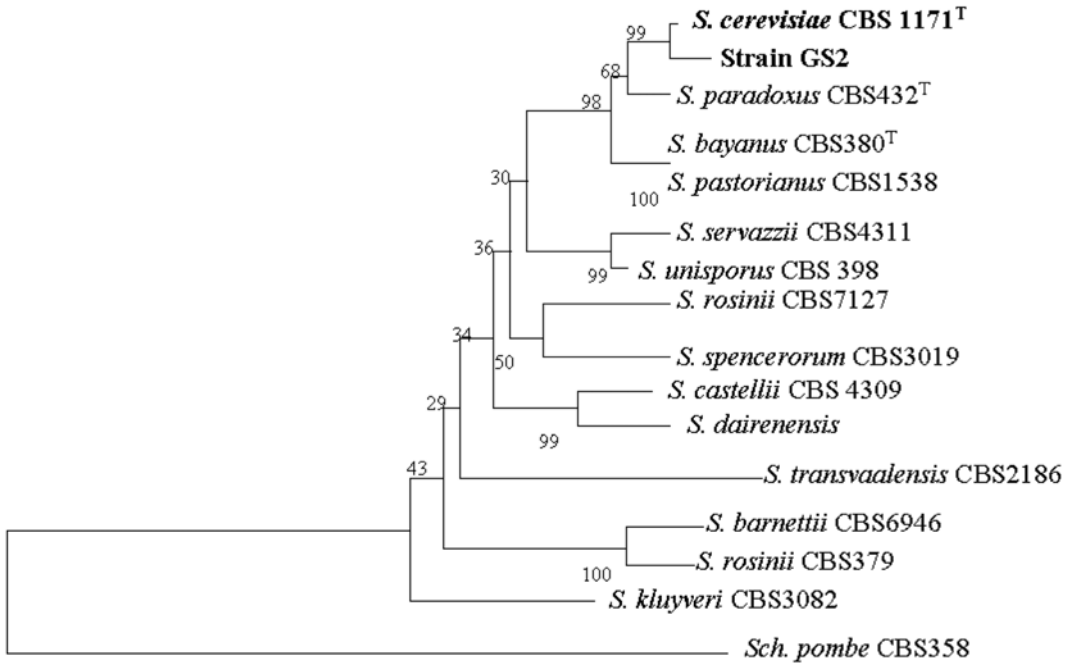

Fig. 1. Phylogenetic relationships among the strain GS2 identified by 26S rDNA D1/D2 sequence constructed using distance matrix analysis (S., *Saccharomyces*).

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4. Cultivation

The culture medium was sterilized for 20 min at 121 °C. The first preculture (50 ml seed medium in 250 ml shake flask) was inoculated with a single colony, cultivated on a rotary shaker at 30 °C for 24 h, and then inoculated in a 5 *l* fermentor with initial working volume of 2 *l* (with 10% [v/v] inoculums). The air flow was maintained as 4 $l \cdot h^{-1}$ and the agitation speed was set as 600 rpm. Culture temperature was kept at 30±0.5 °C and pH at 5.1±0.1 with 6.5 mmol·l^{-1} ammonia. The termination of fed-batch culture was easily recognized by the increase of pH.

5. Experiments about Sodium Selenite Addition in Flask

A flask experiment was performed in a 250 ml flask containing 20 ml YEPD medium after inoculating with 5 ml of seed broth, which was collected from different phase of growth and adjusted to 50 g·l^{-1} after being washed with sterilized saline water. The temperature, agitation rate, and growth period employed in culture were fixed at 30 °C, 180 rpm, and 8 h, respectively.

6. Analytical Methods

Five milliliters of the culture medium was centrifuged at 4,000 rpm for 10 min and washed twice with the deionized water. The resulting solid was dried at 105 °C with a vacuum drying oven for 4 h to obtain dry cell for the estimation of dry cell weight (DCW, g·l^{-1}). At the same time the sample's optical density (OD) was measured spectrophotometrically at 660 nm. Then a standard curve equation [DCW (g·l^{-1})=0.29×dilution multiple×OD, the value of OD was adjusted from 0.3 to 0.8 through dilution] was calculated.

Glucose concentration was measured by a glucose analyzer (SBA-40, Shandong Academy of Agriculture and Science, China).

Measurements of total Se accumulation were performed by atomic absorption spectrophotometry (AAS). Cells were harvested by centrifuged for 5 min at 4,000 rpm and washed three times in deionized water to remove surface-bound Se. Cells were then digested for the total Se estimation according to the method of Suhajda et al. [7]. Inorganic Se was extracted from cells according to the method of Yin et al. [13]. Se content was measured by using an atomic absorption spectrophotometer (SPECTRAA-55B, Varian, American). Organic Se yield was calculated from the difference between the total Se yield and inorganic Se yield. Se content was calculated by Se concentration against DCW.

RESULTS AND DISCUSSION

1. The Test of Fermentation Capability about GS2

Glucose is the favorite carbon source for *S. cerevisiae* to utilize, but the high glucose concentration leads to catabolite repression irrespective of oxygen concentration. So the glucose concentration in the medium is critical for the biomass production. Fed batch culture is the preferred mode because this process can avoid substrate inhibition and improve cell density. Indirect feedback control schemes, such as DO-stat [14], ethanol evolution rate control [15] and direct substrate feedback control have been successfully applied to achieve high cell density cultivation. Although these strategies can manage nutrient supply with demand and vary with the metabolic condition and phase of fermentation operation, the key principle is to control the productivity of DCW and cell yield on substrate to achieve the maximization of the target products.

As indicated in Fig. 2, glucose feeding started at 10 h when the glucose concentration in medium was below 0.2 g·l^{-1}, and this figure was kept at the same level till the end of fermentation. The glucose feeding rate increased along with DO decline from 10 h to 18 h. However, due to oxygen limitation, the glucose feed rate reached a relatively stable value after 18 h. The ethanol concentration reached the maximum at 9.5 h, and gradually dropped to a low level until

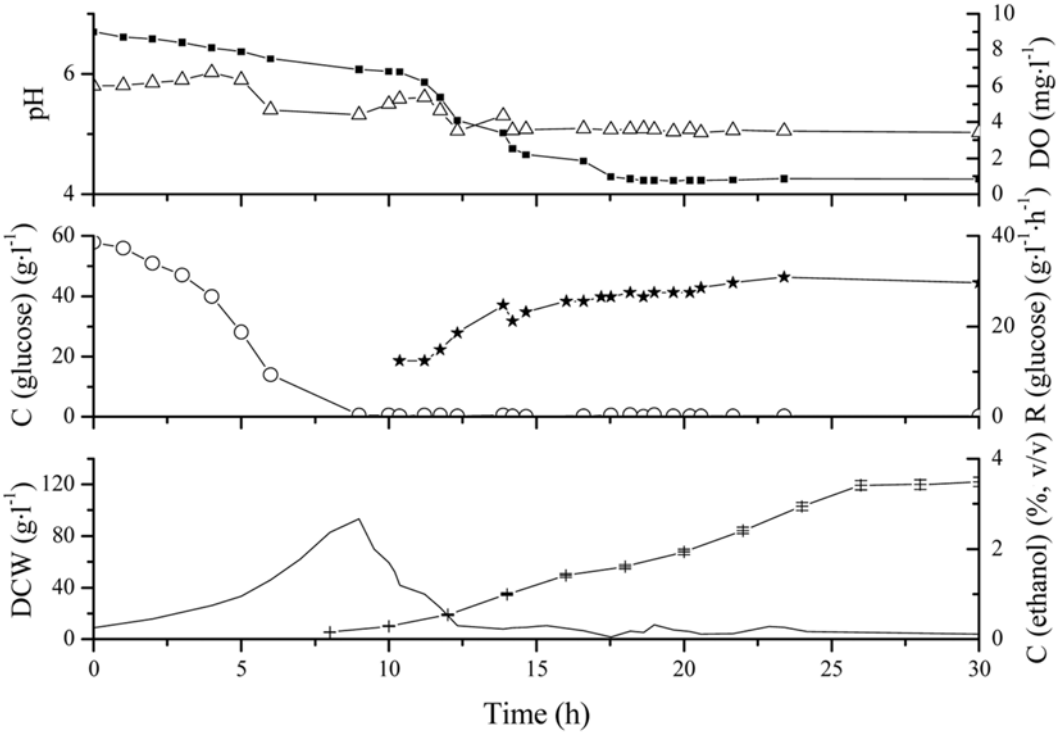

Fig. 2. Time-courses of GS2 fermentation. C (glucose) indicates glucose concentration; R (glucose) indicates glucose feed rate; C (ethanol) indicates ethanol concentration. The first section shows (△) pH and (■) DO; the second section shows (○) C (glucose) and (★) R (glucose); the third section shows (+) DCW and (−) C (ethanol).

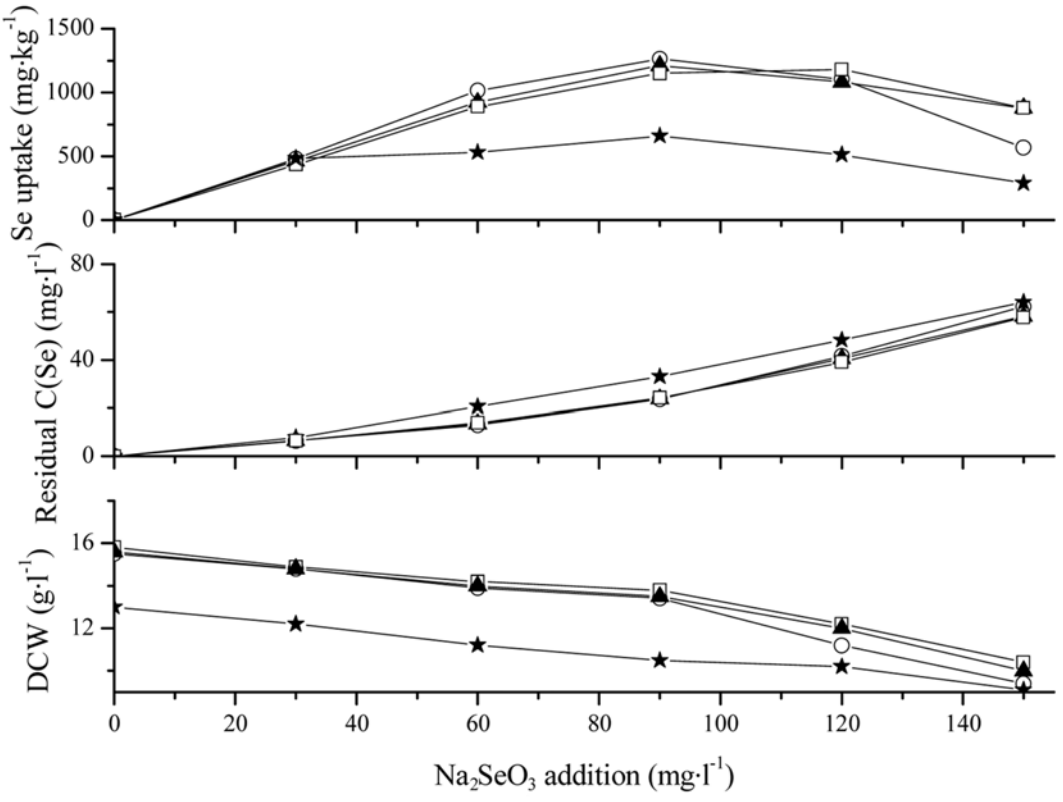

Fig. 3. The effect of Na_2SeO_3 addition on Se accumulation in different growth phases, shown in Fig. 2. (▽) indicates the seeds collected at the earlier exponential phase, 13 h; (▲) indicates the seeds collected at the mid exponential phase, 18 h; (–) indicates the seeds collected at the late exponential phase, 23 h; (★) indicates the seeds collected at the stationary phase, 28 h. Each flask experiment was repeated three times. The total experiment was repeated for reproducibility.

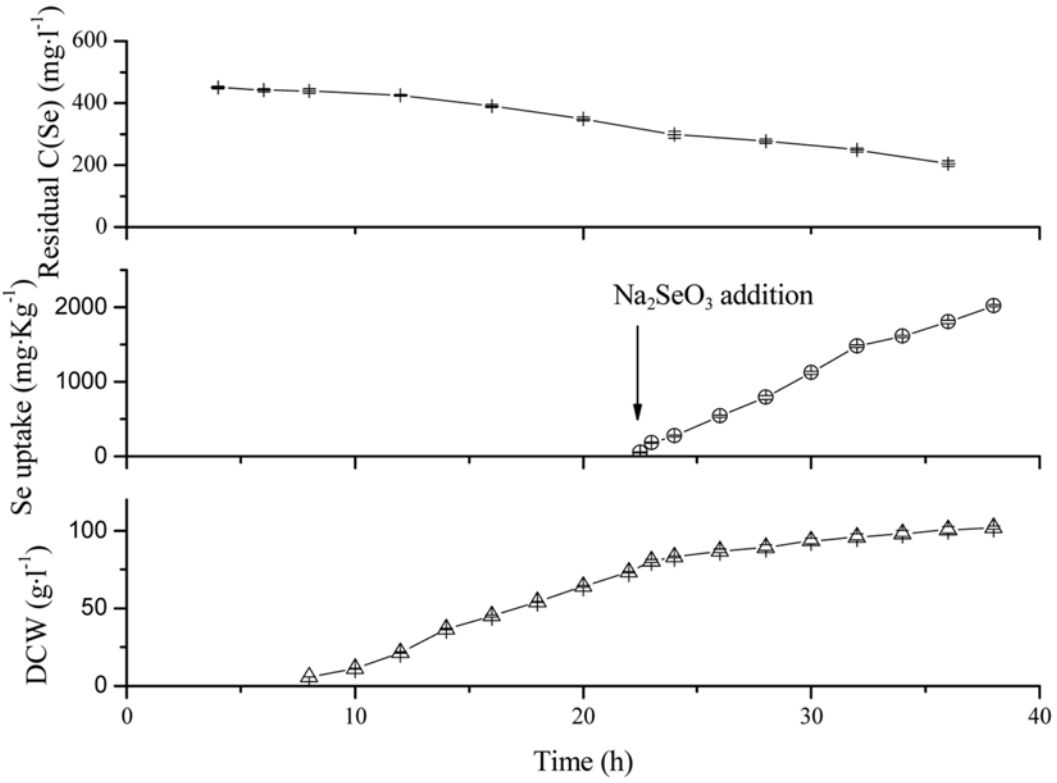

Fig. 4. The effect of Na_2SeO_3 single addition on Se accumulation in a 5 *l* fermentor (8) indicates DCW; (–) indicates Se uptake; (+) indicates Residual C(Se) which represents residual Se concentration in the broth. The error bars represent standard error.

13 h. After 13 h, the ethanol content was almost kept as a constant. DCW reached 122±0.5 g·l^{-1} and yielded 0.44 g DCW·(g glucose)$^{-1}$ after 30 h cultivation. Thus glucose feeding, which was kept to retain a low residual glucose concentration in the medium, was an appropriate strategy for GS2 cultivation.

2. Effect of Na_2SeO_3 Addition on Se Accumulation in Different Growth Phases

High concentration of sodium selenite in the culture medium had a strong inhibitory effect on the growth of yeast and resulted in the red color of the product [7]. It seemed that Se accumulation depended on the biomass density, the phase of growth, treatment time and sodium selenite concentration. Suhajda et al. [7] and Demirci et al. [9] preferred to a single addition of sodium salt during the exponential growth phase.

As shown in Fig. 3, the 50 g·l^{-1} seeds collected at the earlier, mid and late exponential phase (13 h, 18 h, 23 h, respectively) and at the stationary phase (28 h), were inoculated at 20% (v/v) into YEPD medium. Addition of Na_2SeO_3 was toxic to cells because the DCW declined obviously in all the cultures inoculated with seed strains collected from the different growth phases. Seeds from the later exponential phase were least suppressed compared to seeds from three other phases. Se uptake increased with Na_2SeO_3 addition ranged from 30 to 90 mg·l^{-1} and declined when Na_2SeO_3 concentration was higher than 90 mg·l^{-1}. Meanwhile, residual Na_2SeO_3 concentration was gradually rising. The maximal Se uptake reached 1,263±28 mg·Kg^{-1} (the proportion of organic Se was 88%) with addition of 90 mg·l^{-1} Na_2SeO_3 and seed came from the later exponential phase. The optimal pattern of Na_2SeO_3 addition was 9 mg Na_2SeO_3 against 1 g DCW at the late exponential phase and the mean specific Se consumption rate was 157 mg·Kg^{-1}·h^{-1} according to our performances as list in Fig. 3.

3. Fed Batch Culture for Se-enriched Yeast

From the flask experiments mentioned above, we conclude that 9 mg Na_2SeO_3 against 1 g DCW should be added during the late exponential growth. So the scaling up experiments were carried out in a 5 *l* fermentor. As indicated in Fig. 4, the single dose addition of Na_2SeO_3 was performed at the late exponential phase. Se was accumulated gradually with the increase of DCW. Over 38 h of fermentation, the increase of pH indicated autolysis of the cells. The final biomass reached 102±0.4 g·l^{-1} and the Se uptake level achieved 2,020±13 mg·Kg^{-1} (the proportion of organic Se was 91%). Compared with the culture without the addition of Se (Fig. 2), the growth of cells was suppressed and the mean specific growth rate from 23 h to 25 h only reached 0.03 h^{-1}, which was much lower than 0.11 h^{-1} in Fig. 2. However, the uptake of Se, characterized by mean specific Se consumption rate, was 126 mg·Kg^{-1}·h^{-1} in the scale up experiment. This indicated the conversion of organic Se followed a similar way as flask experiments did. At the end of fermentation, residual concentration of Se was 205 mg·l^{-1} and the total conversion of Se was up to 62%.

CONCLUSION

Organically bound selenium yeast was produced with high cell density fermentation. The strain GS2 was tested and 122±0.5 g·l^{-1} dry cell weight was obtained after 30 h cultivation in a 5 *l* fermentor.

Then the optimal pattern of Na_2SeO_3 addition was 9 mg Na_2SeO_3 against 1 g DCW at the late exponential phase. To balance the Se incorporation and optimum growth of the yeast, the fermentation was divided into two stages. The first stage was the glucose feeding to achieve the maximum biomass via controlling the concentration of the residual glucose lower than 0.2 $g \cdot l^{-1}$, and the second stage was the conversion of organic Se by the single dose addition of Na_2SeO_3 at the late exponential growth phase to minimize the suppression of the growth. The final biomass reached 102±0.4 $g \cdot l^{-1}$ and the Se uptake level was 2,020±13 $mg \cdot Kg^{-1}$ (the proportion of organic Se was 91%), which exhibited a potential for industrial application.

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