

# Preparation of Organic Selenium Yeast by Fed-Batch Fermentation

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## Abstract

Selenium is highly proper prepare essential element for the development of mammals and its incorporation in yeast could serve to Selenium enriched yeast. Production of Selenium (Se) enriched yeast was studied by fed-batch fermentation technology using synthetic nutrients to minimize the influence of sulfur and glucose used as the carbon sources. The effect of Selenium (Se) on yeast growth was also evaluated by employing. Se-adapted yeast cultures in continuous fermentation. Fed-batch fermentation with a total of 100 ppm of Sodium selenite in the medium ( $\text{Na}_2\text{SeO}_3$ ), yielded  $32.09 \text{ g L}^{-1}$  of dry yeast cell biomass containing  $3758 \pm 6.5$  ppm of organic Se. The resultant product had the Methylene Blue Reduction Test (MBRT) timing of  $> 25$  min, indicating the highest accumulation of organic Se. The elemental analysis showed the sulfur content was reduced by 37% in selenium yeast (Se-yeast) than the normal yeast produced without Se supplementation. There was a reduction in the experimental biomass yield of Se-yeast by  $18 \text{ g L}^{-1}$  than the theoretical biomass yield in response to the Se addition. A morphological analysis of the normal and Se enriched yeast cells using scanning electron microscopy indicated a reduction in cell size by  $0.88 \mu\text{m}$  in response to Se supplementation.

**Keywords:** Se-adapted yeast, MBRT, organic selenium, fed-batch fermentation, oxygen

Yeast is one of the most important industrial products among the other products like antibiotics, vitamins, industrial enzymes and chemicals based on the volume of sales and utilization (Doramola and Zampraka, 2008). *Saccharomyces cerevisiae* has been used for several centuries in brewing and bread making industry. The yeast fermentation involves the conversion of sugary substrates into ethanol, carbon dioxide and biomass. Under high concentration, sugar is metabolized into ethanol and  $\text{CO}_2$ , which affect the yeast cell biomass production. This phenomenon is known as “crab tree effect” (Aiba *et al.*, 1976; Woehrer and Roehr, 1981). In fed-batch culture, the inhibition due to the substrate or catabolic repression is overcome by intermittent feeding of the substrate. During

fermentation, the yeast cells convert the inorganic selenium (Se) into organic selenium by reducing the Se compounds, thereby making it more bio-available and less toxic to the animals and human beings (Korhola *et al.*, 1986). In mammals, Se is highly essential for the healthy development, as it is translationally incorporated into the amino acid, selenocystein into selenoproteins to perform specific and essential functions in the organism (Diwadkar-Navsariwala *et al.*, 2006; Kryukov *et al.*, 2003; Rayman, 2000). *Saccharomyces cerevisiae* has been successfully enriched with different trace minerals including Se (Korhola and Edelmann, 1986; Hegoczki *et al.*, 1995; Czauderna *et al.*, 1996).

Because of its chemical similarity with sulfur (S), Se follows the same metabolic pathways as S. This concept was strengthened by the discovery that plants and bacteria metabolize Se to the organic selenomethionine and selenocysteine (Burnell and Whatley, 1977). Uptake of inorganic Se from the culture medium and its conversion into selenomethionine by yeast (*S. cerevisiae*) was reported as early as 1961. Though 6000 ppm of Se could be accumulated in a yeast cell based on the methionine content theoretically, commercially available yeast in the market contains only 500 – 2000 ppm of Se (Schrauzer, 2006). Ouerdane and Mester (2009) have suggested that the global replacement of methionine with Se-methionine is possible in yeast cells. The incorporation of inorganic Se may occur through pathways different from S, potentially through non-specific enzymatic residual catalytic activities which might utilize selenides. Food and Drug Administration (FDA, 1987) has permitted the use of Se-yeast as an organic Se source in feed stocks of animals like poultry, beef and dairy cattle.

Yeast cells are capable of proliferating under both anaerobic and aerobic conditions and is capable of utilizing wide range of carbon sources, glucose is the preferred source of carbon for fermentative metabolism (Ponce de leon *et al.*, 2002). A strain of *S. cerevisiae* (strain 1026) tolerant to high concentrations of Se was used in the experiment (Demicri and Pometto, 1999; Rajashree and Muthukumar, 2013 a and b). In this study, we developed a protocol for scaling up the biomass production of yeast enriched with high concentration of Se through fed batch cultivation using glucose as the carbon source. Further, the differences between the calculated theoretical and experimental biomass yield was also compared. In addition, we also evaluated the influence of the Se on yeast cell growth and Se accumulation.

## Materials and Methods

### Culture and Inoculum Development:

Brewer's yeast, *S. cerevisiae* (strain NCYC 1026) obtained from National Collection of Yeast Cultures, United Kingdom was used for the experiment. The stock culture was maintained in Yeast -Malt agar (YMA) slants at 4 °C. Monthly serial transfers of working culture were performed to maintain viability.

**Pre-seed:** Few colonies from the pure culture of *S. cerevisiae* 1026 from *Chloramphenicol Yeast Glucose* slant was transferred to a 50 ml Sabouraud Dextrose broth (SD

Broth - Dextrose 20 g L<sup>-1</sup>, Peptone 10 g L<sup>-1</sup> final pH 5.6 ± 0.2) (Hi-media), and incubated at 30 °C for 18 to 24 hours.

**Seed:** The pre-seed was then, transferred to 450 ml of SD broth and incubated for 18 to 24 hours at 30 °C, in an orbital shaking incubator at 200 g. The optical density of the inoculum was observed to calculate the dry cell weight of yeast and 10 % of inoculum used for the fermentation.

### Fed batch Fermentation

The fermentation experiment was carried out in 10 L laboratory fermentor (Scigenics India) with the working volume of 7 L, equipped with controls for temperature, pH, dissolve oxygen, agitation and foam control. Five liter (5 L) of synthetic medium was prepared in the fermentor and sterilized at 121 °C with 1.2 bar pressure for 15 minutes. The fermentor was cooled down to 30 °C and inoculated with the seed culture and the time of inoculation was recorded as zero hour of fermentation.

### Nutrients

**Initial medium:** The fermentation medium consisted of synthetic nutrients to minimize the presence of sulfur and the composition per liter was as follows: 20 g Glucose, 20 g, NH<sub>4</sub>Cl<sub>3</sub>, 3.0 g KH<sub>2</sub>PO<sub>4</sub>, 0.3g CaCl, 0.3g MgCl, 20 ml Mineral stock (278 mg FeSO<sub>4</sub>.7H<sub>2</sub>O, 288 mg ZnSO<sub>4</sub>.7H<sub>2</sub>O, 80 mg CuSO<sub>4</sub>.5H<sub>2</sub>O, 242 mg Na<sub>2</sub>MoO<sub>4</sub>.2H<sub>2</sub>O, 238 mg CoCl<sub>2</sub>.6H<sub>2</sub>O, 198 mg MnCl<sub>2</sub>.2H<sub>2</sub>O) (Fisher Scientific), 30 ml vitamin stock (10 mg Biotin, 120 mg Calcium pantothenate, 60 mg pyridixin, 120 mg myo-inositol, 120 mg thiamin-HCl), 10 ml amino acid stock (0.1 g adenine, 0.5 g L-arginine, 0.8 g L-aspartic acid, 0.20 g L- histidine, 0.5 g isoleucin, 1 g L- leucine, 0.5 g L- lysine, 0.5 g L- phenylalanine, 0.5 g L-tryptophan, 0.5 g L- tyrosine, 1.4g L-Valine) (Ouerdane and Mester, 2009; Schwenksville and Herber, 1998).

**Feeding and Se supplementation:** Inorganic form of Se was supplemented as sodium selenite (Na<sub>2</sub>SeO<sub>3</sub>) (Sigma-Aldrich) at a concentration of 100 ppm with concentrated glucose solution (150 g L<sup>-1</sup>) and used as a feeding solution.

**Fermentation operating parameters:** The temperature was maintained at 30 °C ±1 and pH at 5 throughout the fermentation. The pH was maintained by the automatic addition of di-ammonium hydrogen phosphate. Aeration was maintained by pumping air at 0.2 to 0.5 vvm (volumes of air per minute per volume of batch) during the growth phase, and agitation from 300–400 rpm to maintain dissolved oxygen in the fermentor broth at 75-90%.

Saturation was measured using Dissolved Oxygen (DO) probe. Silicon anti-foaming agent (10 %) was used for the foam control. Samples were taken at a regular interval of four hours and analyzed for optical density (OD), glucose consumption and microscopic observation. After fermentation, the broth was pasteurized and centrifuged to measure yeast biomass. Later, Se enriched yeast was lyophilized and stored at 4°C, until further analysis.

### Determination of Yeast Biomass

The yeast biomass was determined by measuring the OD using a spectrophotometer (Shimadshu, UV-1700). The standard dilution solutions were prepared with known concentration of yeast cells and observed for optical density at 600 nm in spectrophotometer. This data were plotted against the known dry cell weight (DCW, g L<sup>-1</sup>) values of the yeast biomass. The optical density of the fermentation medium was measured and the DCW was calculated using the equation obtained from the regression curve (R<sup>2</sup>=0.999).

$$M_x(t) = (0.249 \cdot AV) + 0.0015 \cdot V_f(t) \cdot X \quad (1)$$

Where the  $M_x(t)$  is the amount of yeast biomass at the time ( $t$ );  $V_f(t)$  is the volume of the fermentation broth at time ' $t$ ';  $X$ , the dilution factor of the sample;  $AV$ , the absorption value (Doramola and Zampraka, 2008).

### Theoretical and Experimental Results of Yeast Biomass

The theoretical estimation of biomass production was calculated by using the equation (3) Doramola and Zampraka (2008), derived from an analytical solution of Pirt's law of fed batch fermentation (Eqn. 2) (Pirt, 1979). The experimental amount of biomass was obtained from the optical density of the sample obtained from the fermentation broth.

$$r_s = -[\mu/Y_{xs} + M_s] \cdot M_x \quad (2)$$

$$M_x(t) = M_x(0) \cdot e^{\mu_{max} \cdot t} \quad (3)$$

where  $r_s$  is the rate of substrate consumption;  $\mu$ , the specific growth rate (hour<sup>-1</sup>);  $Y_{xs}$ , the yield coefficient;  $M_s$ , the maintenance coefficient;  $M_x$ , amount of biomass;  $M_x(t)$ , the amount of biomass at time  $t$  in the broth during fermentation;  $M_x(0)$ , the initial amount of biomass at the zero hour of fermentation; the  $\mu_{max}$  was assumed as equal to  $\mu$  which was calculated as 0.48 h<sup>-1</sup>; and the  $M_x(0) = 0.50$  g L<sup>-1</sup>.

### Glucose Estimation

The glucose content in the broth was estimated by the Dinitro-Salicylic Acid method (DNS) (Miller, 1972).

### Selenium Analysis

The inorganic and total Se content were analyzed in the final product by iodometric titration (British Pharmacopia). Briefly 5 ml of HNO<sub>3</sub> (25% v/v) and H<sub>2</sub>O<sub>2</sub> (30% v/v) mixture was added to 500 mg of dried yeast sample and digested at 60 °C for 5 min. The digestion process was repeated twice. To the final digestion mixture, 10 ml of urea (10 % w/v) and potassium iodide (10% w/v) were added sequentially and titrated against 0.01N sodium thiosulfate using starch as an indicator.

### Methylene blue reduction (MBRT) method

The Se-yeast product was tested for the extra cellular inorganic Se by the MBRT test (Nagodawithana and Gutmanis, 1985). The Se-yeast product (500 mg) with approximately 100 µg of Se was taken in a screw capped test tube, to which 5 mL of reducing solution [20% (w/v) solution of 1-thioglycerol in 0.20 N phosphate buffer at pH 5.5]. The vial was closed and shaken for 10 seconds. After 3 minutes (at  $t=0$ ), two drops of 2% (w/v) methylene blue solution was added and shaken for 10 seconds, and the solution was left standing for 3 min. The time taken for complete discoloration was recorded as the MBRT time. Sodium selenite purchased from Sigma-Aldrich served as a standard.

### Elemental analysis

Elemental analysis was carried out to compare the percentage of carbon, hydrogen, nitrogen and sulfur content in the end product. An Elementar Vario EL III analyzer was used for the analysis. CHNS analysis: the Se-yeast product (4 mg) obtained from the fed batch fermentation was burnt at 1150 °C. The CHNS elements in a sample were oxidized into CO<sub>2</sub>, H<sub>2</sub>O, NO<sub>2</sub> and SO<sub>2</sub> respectively. The gaseous mixture was then, carried in to a copper tube, in which the all the gases got reduced and absorbed in separate traps and detected by thermo-conductivity detector (Ding *et al.*, 2011).

### Scanning Electron Microscopy (SEM)

The yeast cells with and without Se exposure to different concentrations of Se were observed with SEM for morphological changes. Samples were prepared as per the protocol of McDougall *et al.* (1994) with certain modifications. The yeast cultures grown in SD broth at different concentrations of Se (0 ppm, 50 ppm, 100 ppm, 150 ppm) for 24 hours at 30°C, were harvested by centrifugation. The cells were washed twice with 1X PBS buffer (KCl 0.2 g L<sup>-1</sup>, NaCl 8.0 g L<sup>-1</sup>, KH<sub>2</sub>PO<sub>4</sub> 0.2 g L<sup>-1</sup>,

Na<sub>2</sub>HPO<sub>4</sub> 1.15 g L<sup>-1</sup>) at 10,000 g for 10 min. The cells were then dried in an ethanol gradient of 10 to 100%. The cells were further dehydrated with methanol and finally with 2% glutaraldehyde, and stored in vacuum.

### Statistical analysis

All the data were subjected to analysis of variance (ANOVA) using SPSS software (version 9.2). The means were separated using Duncan's multiple range test (DMRT) when the result of one way ANOVA was significant (p<0.05).

### Results and Discussion

The dry cell weight of seed culture and the fermentation broth at different time intervals were calculated from their optical density by using the equation obtained from the standard curve (Eqn. 1 and Fig. 1). The calculated biomass concentration of yeast seed culture was 5 g L<sup>-1</sup>. Optical density of the broth samples measured at every four hours intervals from zero to 48 hours and the dry cell weight (g L<sup>-1</sup>) was determined. The theoretical biomass yield calculated using Eqn.3, was 50.07 g L<sup>-1</sup> at the 48<sup>th</sup> hour of fermentation. However, the actual biomass calculated from the absorbance values and the final biomass at 48<sup>th</sup> hour was 32.09 g L<sup>-1</sup>. The difference between the actual biomass and theoretical biomass yield was 17.98 g L<sup>-1</sup> (Fig. 2).

Glucose consumption during fed batch fermentation was determined by measuring the glucose concentration in the broth (Fig. 3A). Glucose was consumed by the yeast cells and at 8<sup>th</sup> hour of fermentation, the glucose level decreased. At the 8<sup>th</sup> hour, the yeast cell biomass reached 5.34 g L<sup>-1</sup> and the glucose concentration in broth decreased to less than 0.42 g L<sup>-1</sup>. Therefore, feeding was started based on the glucose consumption. DO levels in fermentation broth

was carefully monitored and noted from zero to 48 hours of fermentation (Fig. 3B). The DO drastically decreased after inoculation and was 1.8 at 12<sup>th</sup> hour. However, the DO started to increase after 44<sup>th</sup> hours of fermentation, an indication of stationary phase. The yeast growth rate also decreased at 44 and 48 hours of fermentation.

At 48 hours, harvest of fed batch fermentation contents revealed a biomass production of 32.09 g L<sup>-1</sup>. Analysis of the supernatant and washed water revealed the presence of Se at concentration of less than 10 ppm. The organic Se in the dry biomass was 3758 ± 6.5 ppm, and the 85.9% of given sodium selenite was incorporated into yeast cells. The MBRT method used for the confirmation of the presence of organically bound Se lasted for more than 25 minutes strongly indicating the presence of only traces of inorganic Se. Therefore, the present study demonstrates the presence of highest concentration of organic Se in yeast biomass. The scanning electron microscopic observation of yeast cells revealed a slight decrease in cell size from 4.77 ± 0.3 μm to 3.88 ± 0.72 μm in response to Se addition (Fig. 4).

An elemental analysis performed to compare the elements such as carbon, hydrogen, nitrogen and sulfur content in yeast and the Se-yeast biomass. The changes in elemental content between the Se-free yeast and Se-yeast were 6.37%, 1.45%, 2.10% and 37.14% in carbon, hydrogen, nitrogen and sulfur respectively.

The fed batch cultivation was applied successfully by maintaining the glucose concentration at appropriate levels to control the catabolite repression and to increase the cell density. Feeding of substrate into the fermenter throughout

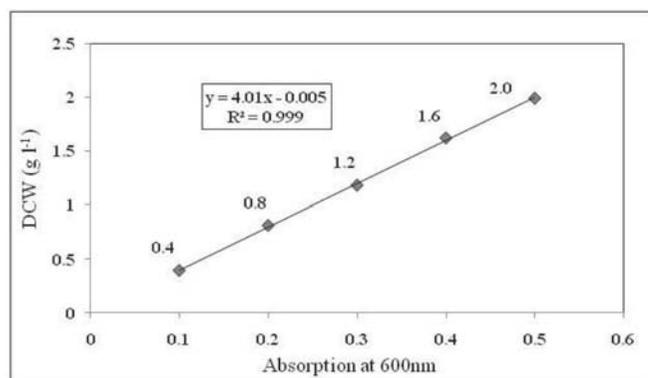


Fig. 1: Absorption values versus dry cell weight (g L<sup>-1</sup>).

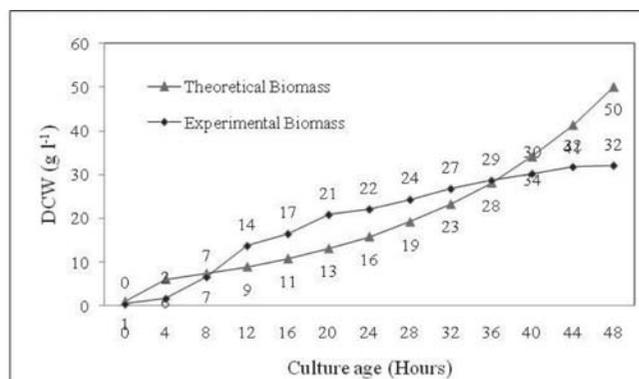
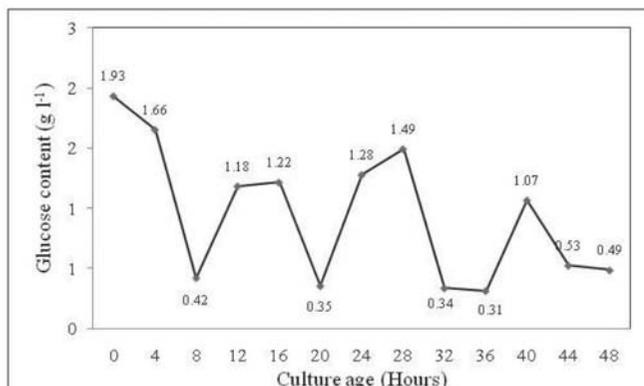


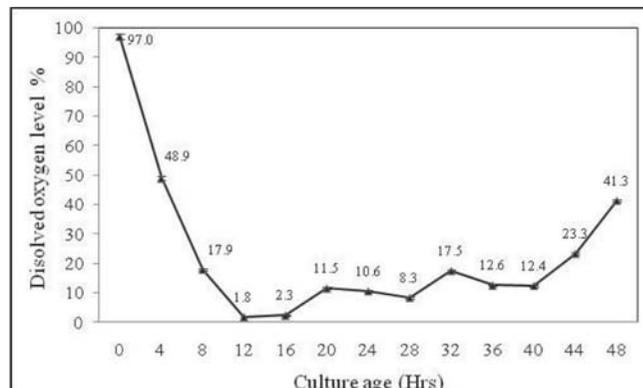
Fig. 2: Theoretical versus experimental biomass yield.

Means ± SD are statistically different according to DMRT (F<sub>7,16</sub> = 1913.74; p < 0.001).

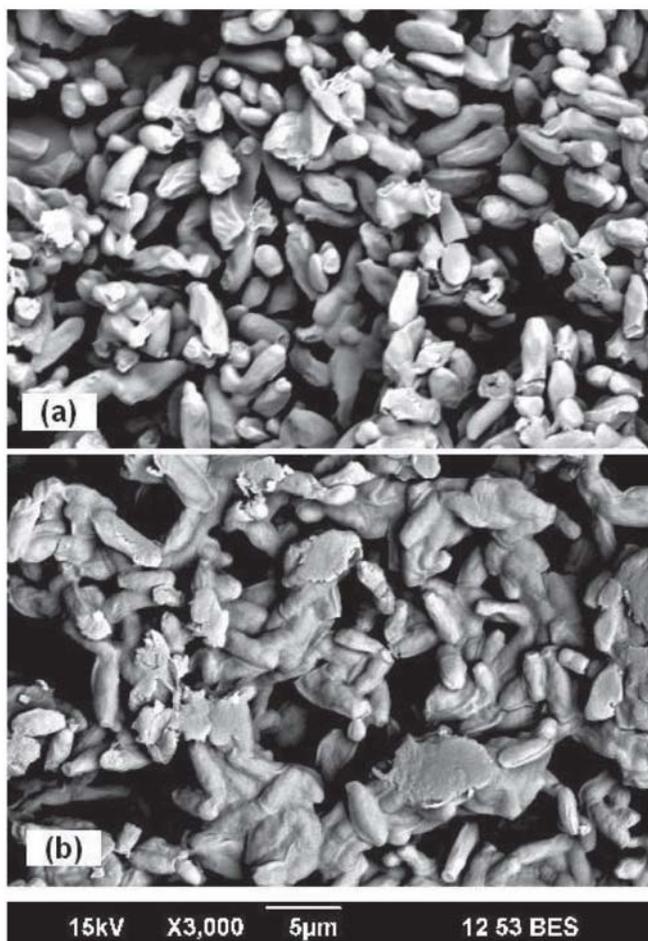
DCW: Dry cell weight.



**Fig. 3A:** Glucose concentration in broth during fed-batch fermentation. Means  $\pm$  SD are statistically different according to DMRT ( $F_{7,16} = 1186.74$ ;  $p < 0.001$ ).



**Fig. 3B:** Dissolved oxygen levels during fed-batch fermentation. Means  $\pm$  SD are statistically different according to DMRT ( $F_{7,16} = 5961.94$ ;  $p < 0.001$ ).



**Fig. 4:** Scanning electron microscopy images of yeast *Saccharomyces cerevisiae*

(a) Before Se supplementation

(b) After Se supplementation

Scale bar: (a) and (b) 5  $\mu$ m

the fermentation to increase *S. cerevisiae* biomass yield was in line with Ghahremani *et al.*, (2009). Thus, the glucose level in the medium was maintained at an optimal level throughout the fermentation starting from 8 hrs of culture when the glucose concentration in the medium declined to sub-optimum level. The inorganic Se was added along with the glucose at a concentration of 100 ppm and was fed continuously throughout the batch. In this study, 641 g of Se-yeast per kilogram of glucose fed (64 %) was obtained, whereas Rosen (1987) reported that under appropriate conditions, the carbon source fed could yield only 50% of dry biomass. A drop in pH of the medium noted during the fermentation process was expected as glucose metabolism results in acid production. However, pH was maintained at 5 by automatic addition of diammonium hydrogen phosphate, which also served as a source of nitrogen and phosphorus (Raj *et al.*, 2003).

The Se-yeast biomass production rate was calculated as 0.66 g L<sup>-1</sup> of broth per hour of fermentation (g L<sup>-1</sup> hr<sup>-1</sup>). The difference between the theoretical and experimental biomass yield was discussed by using the Pirt's law. There was 36% reduction in experimental yeast biomass production than the theoretical biomass yield. The difference in theoretical and actual biomass concentration may be due to the fermentation parameters like yield factors, fermentation efficiency, productivity, production and consumption rates, measured concentrations of biomass, substrates and products and may be influenced by other factors including, evaporation and stripping losses, addition of the acid, base solutions, anti-foam, biomass volume, samples withdrawal which are all known to influence yeast proliferation (Concone *et al.*, 1978; Wang, 1979; Borzani, 2008).

Previous studies have shown that Se enrichment of microbial cells depends on the type of microorganisms involved and the production methods, as has been revealed by various studies. Selenium accumulation of 253 to 500 ppm in *Lactobacillus delbrueckii* (Korhola *et al.*, 1986; Nagodawithana and Gutmanis, 1985), 375 ppm in *L. plantarum* and 407 ppm in *L. casei* (Calomme *et al.*, 1995) have been reported. Demicri and Pometto (1999) reported the Se-yeast (strain ATCC 26787) yield of 0.76 g L<sup>-1</sup> of dry biomass with 687 ppm of Se in dry biomass, with a high MBRT value (26 minutes). Kaur and Bansal (2005) reported Se concentration of 8.69 ppm in *S. cerevisiae* strain MTCC – 1766. Demicri *et al.* (1999) showed that fed batch fermentation with continuous addition of higher concentration of Se could yield 45 g L<sup>-1</sup> of biomass with 2495 ppm of Se in a wild strain of *S. cerevisiae*. In agreement with Dimicri *et al.* (1999), the Se adapted yeast strain in the present study accumulated 3758 ppm of organic Se with the biomass concentration of 32 g L<sup>-1</sup>.

Ouerdane and Mester (2009) characterized the global replacement of methionine by seleno-methionine (SeMet) with >98% substitution, with up to 4940 ppm of SeMet g<sup>-1</sup> of a wild type yeast grown on a SeMet-containing medium, by using synthetic defined media to reduce the influence of sulfur during the growth phase to ensure the maximum Se incorporation. But, the global replacement of Se-methionine is not possible in industrial production. Therefore, in order to get the maximum incorporation of Se in yeast the medium with very low sulfur content was optimized in our study. This was achieved with maximum incorporation of inorganic Se (85.9%) into yeast cells and its subsequent conversion into organically bound Se. The end product yield of fed-batch fermentation was about 32.09 g L<sup>-1</sup> of dry biomass of Se-yeast with an organic Se content of 3758 ppm. The organic Se content in yeast biomass was also confirmed by the high MBRT (> 25 minutes) values. An elemental analysis was carried out to compare the difference between the sulfur ratios in the yeast biomass. Due to the low sulfur content in culture broth and Se replacement, the sulfur content of Se enriched yeast was 37.14 % lower than the normal Se-free yeast. The reduction in sulfur content in Se enriched yeast cells indicates that the majority of sulfur was replaced by organic Se. Nevertheless, the elemental analysis for Se and sulfur has to be subjected to more detailed studies.

Nagodawithana and Gutmanis (1985) reported that the single dose addition of Se would inhibit the yeast growth because of the toxicity. So, Se was fed along with the carbon source

and added slowly till the end of fermentation to reach a concentration of 100 ppm in the final volume of the fermentation medium. This incremental addition of Se along with the carbon source enhanced yeast growth and resulted in optimal uptake and incorporation of Se in yeast biomass.

During fermentation, the samples were drawn at regular intervals for the observation of morphological changes and it was observed in our study the slight deviation in cell size after the Se addition took place. So the cells were subjected to scanning electron microscopy for the measurement of yeast cells before and after Se addition. There was a significant ( $F_{1,18} = 11.987$ ;  $P < 0.003$ ) reduction in cell size in Se enriched yeast. The size of yeast cells were reduced by 0.88 μm and thus it is proven that the Se addition results in the reduction in cell size, which may involved in the reduction of biomass yield, can be subjected for further studies. Dhanjal and Cameotra (2010) also reported a similar reduction in bacterial cell size in response to increasing concentration of selenite in the growth medium. The reduction in cell size and changes in the cell morphology can be attributed to the stress imposed by the high concentrations of the toxic selenite ions on growing cells. In other words, the organisms reduce their cell size and increase their relative surface area for better uptake of the nutrients for survival under environmental stress conditions (Dhanjal and Cameotra, 2010). Selenium is known to interfere with the phosphate uptake mechanism in algae like *Scenedesmus dimorphus* and *Anabaena cylindrica* and thereby, inhibiting the cell growth (Moede *et al.*, 1980).

### Conclusion

The Se-adapted strain yielded 32.09 g L<sup>-1</sup> of biomass with 3758 ppm of Se with an MBRT value of > 25 min., indicating a high organic Se content. Feeding of 1 Kg of glucose by fed-batch fermentation yielded 641 g of yeast, which is an extremely high value. However, the comparison between the theoretical and experimental biomass yield shows that the actual biomass yield was less than the expected biomass yield. The Se addition resulted in a reduction in cell size and biomass yield by 0.88 μm and 18 g L<sup>-1</sup> than the normal and theoretical biomass yield, respectively. The reduction in yeast cell size due to the Se addition ultimately may also have influenced the biomass yield.

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