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Selection of culture medium and conditions for the production of selenium enriched *Saccharomyces cerevisiae*

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Selenium (Se) yeast is a recognized source of organic food-form of Se and it plays a vital role in animal and human nutrition. The organic Se supplementation in the form of yeast has been shown to have beneficial effects on growth, immune status and reproduction in many animal species, thereby improving the productivity and economical benefits in livestock production. Subsequently, strategies to supplement animal feeds with Se yeast have led to the development of industrial production of Se yeast. The aim of the present study was to improve the yeast biomass production measured as dry cell weight (DCW) and Se enrichment of yeast cells through optimization of the culture conditions and culture media. The culture conditions were optimized by the shake flask experiments. Maximum cell density (2.93 g/LDCW) was observed at pH 5 to 5.5, at 30 °C (2.88 g/L DCW). Significantly higher DCW was recorded when glucose was used as the carbon source (3.09 g/L DCW). The media optimization study carried out in fermentors with five different media (defined medium–A, defined medium-B, synthetic medium, rich medium and industrial medium) showed that the synthetic medium yielded maximum yeast biomass (12.8 g/LDCW) followed by rich medium (11.7 g/L DCW) and defined medium B (10.5 g/LDCW). The Se accumulation was also significantly higher in synthetic medium (2718.3 ppm), followed by industrial medium (2457.7 ppm) and defined medium-B (2251.3 ppm). The methylene blue reduction time (MBRT) was very high (>15 m) in synthetic medium indicating the highest accumulation of organically bound Se, and MBRT was moderate for defined medium-A and industrial medium (<10 m).

Key words: *Saccharomyces cerevisiae*, synthetic medium, carbon source, selenium enrichment, methylene blue reduction time (MBRT).

INTRODUCTION

Selenium (Se), the important dietary trace element for both animals and humans, was discovered by Berzelius in 1817. A biologically active form of Se was found in 1973, when glutathione peroxidase was identified as a very potent antioxidant protecting the body from damage due to oxidation by free radicals. The involvement of Se in the metabolic processes is proved by the presence of Se as a part of cellular glutathione (Schwarz and Foltz, 1957). Some of the detrimental effects of Se deficiency include reversible cardiomyopathy, known as Keshan disease, infertility and arthritis in humans. Generally, food-based approaches are considered for preventing Se

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Abbreviations: Se, Selenium; **YMA,** yeast malt agar; **SDB,** Saboroud dextrose broth; **MBRT,** methylene blue reduction time; **DCW,** dry cell weight.

deficiency. Enhancing the intake of any biological forms of Se, reduces the risk of cancer through support of anti-tumorigenic Se-metabolites (Combs, 2000). It has become increasingly evident that dietary Se plays a significant role in reducing the incidence of lung, colo-rectal and prostate cancer in humans (Danielle et al., 2004). However, excess Se can cause chronic selenosis manifested by brittle hairs and nails (Wada et al., 1995).

Recently, Se has emerged as a popular nutritional supplement. The organic form of Se provided by Se yeast has been shown to differ in bioavailability and metabolism compared with inorganic (for example, selenate, selenite) forms of dietary Se (Mahan and Kim, 1996). Further, retention of organically bound Se in the body is longer than that of inorganic Se (Foster and Sumar, 1997). Se enriched yeast is one of the most widely used Se supplement. Yeast is well known for its high protein content and more Se can be incorporated into yeast cells by replacement of sulfur in the sulfur containing proteins. Industrial production of Se enriched yeast is more manageable and economical than the production of Se-enriched plants (Ponce de Leon et al., 2002). Indeed, inorganic, Se (IV) is converted into compounds containing Se in its reduced forms (organic Se). Thus, organic Se has an improved nutritional quality as it is much less toxic and more bioavailable than the inorganic Se (Karhola et al., 1986).

Feeding of Se enriched yeast to animals has been shown to have beneficial effects on the immune status, growth and reproduction in animals (Dlouha et al., 2008). The requirement of Se yeast as a feed additive has become prominent in livestock production. As per the report of the “National Diary Development Board” (NDDB), the total livestock population in India was 1708 Million which included cattle, goat, sheep, poultry, among others (NDDB, 2007). The ever increasing livestock population in India increases the demand for the feed production and the value added feed additives. Se-yeast is one of such value added feed additives. Hence it is an important area of research which prompted us to undertake this study.

Previous studies have analyzed the amount of Se that could be incorporated into yeast cells. In those studies, the amount of Se that could be incorporated into yeast cells using sodium selenate or sodium selinite ranged between 500 to 3000 ppm (Karhola et al., 1986; Nagodawithana et al., 1985; Demirci et al., 1999). Recently, Marinescu and Antoneta (2011) reported Se yeast production using brewer’s yeast (*Saccharomyces uvarum*) cultivated in the malt wort and sparge water industrial nutrient medium enriched with sodium-selenite. They noted that the yeasts were capable of accumulating large amounts of Se mainly during the growth phase of the cells. Supplementation of the culture medium with 30 to180 µg/ ml sodium selenite resulted in total Se accumulation in the range of 300 to 2200 ppm in yeast cells (Marinescu and Antoneta, 2011).

Previous studies reporting the development of Se enriched yeast involved different media compositions with various ingredients like pure synthetic chemicals, complex nutrients and industrial byproducts (Nagodawithana et al., 1985; Ouerdane and Mester., 2008). The production of Se yeast is influenced by a number of factors. For example, high concentration of Se in culture media inhibits the growth of yeast cells (Nagodawithana et al., 1985).

In addition, some media ingredients such as peptone, yeast extract and other compound nutrients that contain sources of sulfur also interferes with the uptake of Se by yeast cells (Ouerdane and Mester, 2008). The physical parameters such as pH, temperature and dissolved oxygen level of the media are the most important parameters influencing the Se incorporation into yeast cells (Suhajda et al., 2000). The purpose of this present study was to develop a method for the preparation of Se yeast with high Se content and to increase the yield of yeast biomass through selection of culture media and culture conditions.

MATERIALS AND METHODS

Micro organism and culture maintenance

Saccharomyces cerevisiae NCYC 1026 was obtained from the National Collection of Yeast Cultures, United Kingdom. The strain was maintained on Yeast-Malt agar (YMA) slants stored at 4°C and transferred monthly on to fresh slants and incubated at 30°C for 2 days to maintain viability.

Media for culture maintenance and optimization

The mother stock and working cultures were maintained on Yeast malt agar (YMA). Saboroud dextrose (SD) broth (Hi-media), defined medium –A, defined medium-B, synthetic medium, rich medium and industrial medium were used for media optimization in shake flask experiment (Table 1). All media were prepared with chemicals of laboratory-grade using distilled water (George and Herber, 1998).

Culture conditions for yeast biomass production

Shake flask experiments were conducted to optimize the cultivation conditions. 50 ml of SD broth in 250 ml conical flasks were sterilized at 121°C, 15 lbs pressure for 15 min. The flasks were inoculated with the pre-inoculum volume of 2% to give an initial yeast cell concentration of 0.1 g/L. The cultures were incubated for 18 h in an orbital shaker incubator (Remi Laboratory Instruments, Mumbai, India) operated at 200 rpm. The levels of pH (4.5 to 7.5), temperature (27-33°C) and different carbon sources (glucose, sucrose, molasses, glycerol, maltose, starch, fructose) were used in the optimization studies. For the selection of carbon sources, all prepared media had the same chemical constituents except the carbon source. The optimization studies were carried out by varying one parameter, while keeping the others constant.

Selection of media for Se enrichment:

Media selection for high Se enrichment involved five different media used for the cultivation and for Se enrichment. The respective media were prepared in duplicates and sterilized at 121°C for 15 min.

Table 1. Composition of media used for inoculum development and shake flask experiments.

Media	Composition (g/L)
Yeast malt agar	Glucose, 10.0 g; Yeast extract, 3.0 g; malt extract, 3.0 g; peptone, 5.0 g; a gar, 20.0 g
SD broth	Glucose, 20.0 g; Peptone, 10.0 g
Defined media -A	Glucose, 20.0 g;NH_4Cl, 9.0 g; M gSo_4,1.0 g; KCl, 1.0 g; Thiamin HCl, 0.1 g; Peptone, 15.0 g
Defined media -B	Glucose, 20.0 g; $(NH_4)_2SO_4$, 3.0 g; KH_2PO_4, 3.0 g; $(NH_4)_2SO_4$, 1.0 g; CaCl, 0.3 g; M gCl, 0.3 g; Yeast extract, 10.0 g
Synthetic media	Glucose, 20.0 g; NH_4Cl, 3.0 g; KH_2PO_4, 3.0 g; CaCl, 0.3 g; M gCl, 0.3 g; Mineral stock, 20.0 ml; Vitamin stock, 30.0 ml; Amino acid stock, 10 ml
Trace mineral Stock (10x)	$FeSO_4.7H_2O$, 0.278 g; Zn $SO_4.7H_2O$, 0.288 g; $CuSO_4.5H_2O$, 0.080 g; $Na_2MoO_4.2H_2O$, 0.242 g; $CoCl_2.6H_2O$, 0.238 g; $MnCl_2.2H_2O$, 198.0 g
Vitamin stock (10x)	Biotin, 0.01 g; Calcium Patothenate, 0.12 g; Pyridixin, 0.6 g; Myo-Inositol, 0.12 g; Thiamin-HCl, 120 g
Amino acid stock (10x)	Adenine, 0.1 g; L-Arginine, 0.5 g;L-Aspartic acid0.8 g; L- Histidine, 0.2 g; L- Isoleucin, 0.5 g; L- Leucine, 1 g; L- Lysine, 0.5 g; L-Phenylalanine, 0.5 g; L-Tryptophan, 0.5 g; L- Tyrosine, 0.5 g; L-Valine, 1.4 g;
Rich media	Dextrose, 40.0 g; Peptone, 20.0 g; Yeast extract, 10.0 g
Industrial media	Molasses, 20.0 g; Corn steep liquor, 20.0 g; Yeast extract,10.0 g

Inorganic Se in the form of sodium selenite (Na_2SeO_3) was added to all media at an equal concentration of 50 ppm. Seed culture was prepared by growing yeast in SD broth in Erlenmeyer flasks shaken overnight at 200 rpm. Under aseptic conditions the flasks were inoculated with the inoculum volume of 2% to give an initial cell concentration of 0.05 g/Land incubated in orbital shaking incubator for 24 h. The pH and temperature were maintained as per the optimization studies for both seed preparation and fermentation experiments. Samples were collected at the 24^{th} hour of fermentation for the determination of dry cell weight (DCW) (g/L), and Se uptake.

Estimation of Se uptake

Samples from the fermentation systems were centrifuged thrice in a refrigerated centrifuge (Remi, Cooling centrifuge, Mumbai, India) at 5000 *g* for 5 min each. At the end of the centrifugation, the supernatant was collected and replaced by deionized water, homogenized with yeast biomass and centrifuged again to remove the impurities and traces of inorganic Se. The end product, cleaned yeast samples were dried at 40°C overnight to a constant weight. The dried yeast and the supernatant samples were estimated for total Se and inorganic Se, respectively. The organic Se content of the Se yeast was calculated as the difference between the total Se and the inorganic Se (Yin et al., 2009). The Se-yeast samples were digested with nitric acid and dispersed to analyse the total mineral content. This procedure is designed to determine the percentage of total Se in a yeast sample (AOAC method, 1995).

Methylene blue reduction time (MBRT) method

The MBRT method was used to determine the type of Se in yeast biomass. The MBRT time determined for pure inorganic Se salts such as sodium selenite or sodium selenate was less than a minute. Always, a high MBRT indicates the presence of higher concentrations of organically bound Se. Therefore, a MBRT of more than 15 to 20 min indicates the presence of sufficiently low extra cellular Se (Nagodawithana et al., 1985; Demirci and Pometto, 1999).

Analytical methods

Determination of dry cell weight (DCW)

In order to determine the dry cell weight (DCW), 10 ml broth was centrifuged at 8,000 *g* for 10 min in a refrigerated centrifuge (Remi Laboratory Instruments, Mumbai, India) and washed twice with deionized water to remove the extracellular debries. The yeast cream was dried at 105°C for 2 h and weighed to estimate the DCW.

Se analysis

The inorganic and total Se content were analyzed by iodometric titration (British Phamocopia). Briefly, 5 ml of HNO_3 (25% v/v) and H_2O_2 (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 0.01 N sodium thio sulfate using starch as an indicator.

MBRT method

The Se-yeast product was tested for the presence of extra cellular inorganic Se by the MBRT test (Nagodawithana et al., 1985). 500 mg of Se yeast product containing approximately 100 µg of Se was taken in a screw capped test tube to which 5 mL of reducing solution (1-thioglycerol 20% (w/v) in 0.20 N phosphate buffer at pH 5.5) was added. The vial was capped and shaked for 10 s. After 3 min (at = 0), two drops of 2% (w/v) methylene blue solution was added and shaken for 10 s and the solution was left standing for 3 min . The time taken for discolorization was recorded as the MBRT time.

Statistical analysis

Significance of variations among data was statistically determined with IRRISTAT software (International Rice Research Institute, version 3/93). Duncan's multiple range test was applied when the one-way ANOVA showed obvious differences ($p<0.05$).

RESULTS AND DISCUSSION

Optimization of cultivation conditions

Media pH ($F_{6,10}$ = 23.12) and temperature ($F_{6,10}$ = 46.09) significantly ($p < 0.01$) influenced the yeast DCW. High DCW (2.93 g/L) was achieved in pH 5.0 and 5.5 which was 7.35% higher compared to DCW in pH 4.5 and 6.93 to 38.86% higher than pH 6.0 to 7.5 (Figure 1A). Maximum yeast biomass (2.88 g/L) was recorded at 30°C and the least biomass (2.27 g/L DCW) production occurred at 33°C. Gradual increase in temperature from 27 to 30°C increased yeast biomass, but a further incre-ase reduced DCW production by 22.55 to 26.87% (Figure 1B). The sequence of other carbon sources that sup-ported yeast biomass production was in the order of maltose > molasses> sucrose > fructose> glycerol>starch (Figure 1C).

Culture conditions for *S. cerevisiae* were optimized based on the DCW production in shake flask experiments. The carbon source is the major nutrient which contributes to the multiplication of yeast cells. The glucose supplemented medium yielded the highest yeast biomass (3.09 g/L) which was 18 to 145% higher compared to the other carbon sources tested. Although, *S. cerevisiae* is capable of proliferation under both anaerobic and aerobic conditions and can use wide variety of compounds as carbon sources. Glycerol and starch did not adequately support the growth of yeast cells and the DCW were 1.55 g/Land 1.26 g/L, respectively. Several studies (Dickson and Schweizer, 1999; Ponce de Leon et al., 2002) have reported the inhibitory effect of glycerol on yeast cell growth. As glucose appeared to be preferred for fermentation metabolism (Pone de Leon et al., 2002), we used glucose as the main carbon source in subsequent experiments.

Optimization of culture media

The variations in the biomass yield in the various media indicated significant ($F_{4,10}$ = 56.34; $P<0.01$) influence of culture medium on yeast biomass production. Highest biomass production (12.8 g/L) was obtained with synthetic media followed by rich media (11.7 g/L) and defined media-B (10.5 g/L). The lowest biomass yield (9.6 g/L) occurred in defined media-A. The industrial media yielded about 10.2 g/Lof yeast biomass (Table 2).

Based on the results of the shake flask experiments, the culture conditions were optimized and used for the media optimization. Five types of media were screened for their ability to proliferate *S. cerevisiae*. The defined media A and B contains different nitrogen sources like ammonium sulfate, ammonium chloride, peptone and yeast extract. To achieve the maximum uptake of Se by yeast cells, all aspects of the yeast media compositions were optimized including sources of sulfur and Se. The synthetic medium is formulated based on this objective and it has very less or no sulfurs (Ouerdane and Mester., 2008), whereas the rich medium has glucose, yeast extract and bacteriological peptone. As a whole, the supplementation of vitamins, trace minerals and amino acids used in synthetic media has improved the production of yeast biomass. The industrial medium contains industrial byproducts, which is very economical and efficient source for the yeast biomass production.

Se uptake in yeast biomass

The amount of Se accumulated by yeast cells significantly ($F_{4,10}$ = 3317.09; $p < 0.001$) differed among the various media tested. Maximum accumulation of Se was obtained with synthetic medium (2718.3 ppm), followed by industrial medium (2457.7 ppm), defined medium-B (2251.3 ppm), defined medium-A (1980 ppm) and rich medium (1640 ppm). The DCW (g/L) and Se accumulation in different culture media are listed in Table 2.
Suhajda et al. (2000) reported a Se accumulation of 1200 to 1400 µg/g dried baker's yeast using a culture theoretically a yeast cell can incorporate around 6000 ppm of Se, full replacement of methionine by seleno-methionine is not possible (Schrauzer, 2006).

Although, the second highest yeast biomass yield was recorded in the rich medium, the Se accumulation in yeast cells was least in this medium. This may be due attributed to the presence of complex nutrients like peptone and yeast extract that might have inhibited the Se incorporation into the yeast cells as noted by Ouerdane and Mester (2008). The Se accumulation in rich media (1640 ppm) was 21 to 66% lower when compared to other media. Although, the DCW production in defined media B and industrial media was 11.42 and 14.75% lower than the rich medium, the Se incorporation in yeast cells in defined media B and industrial media was 65.75 to 49.86% higher than the rich medium.

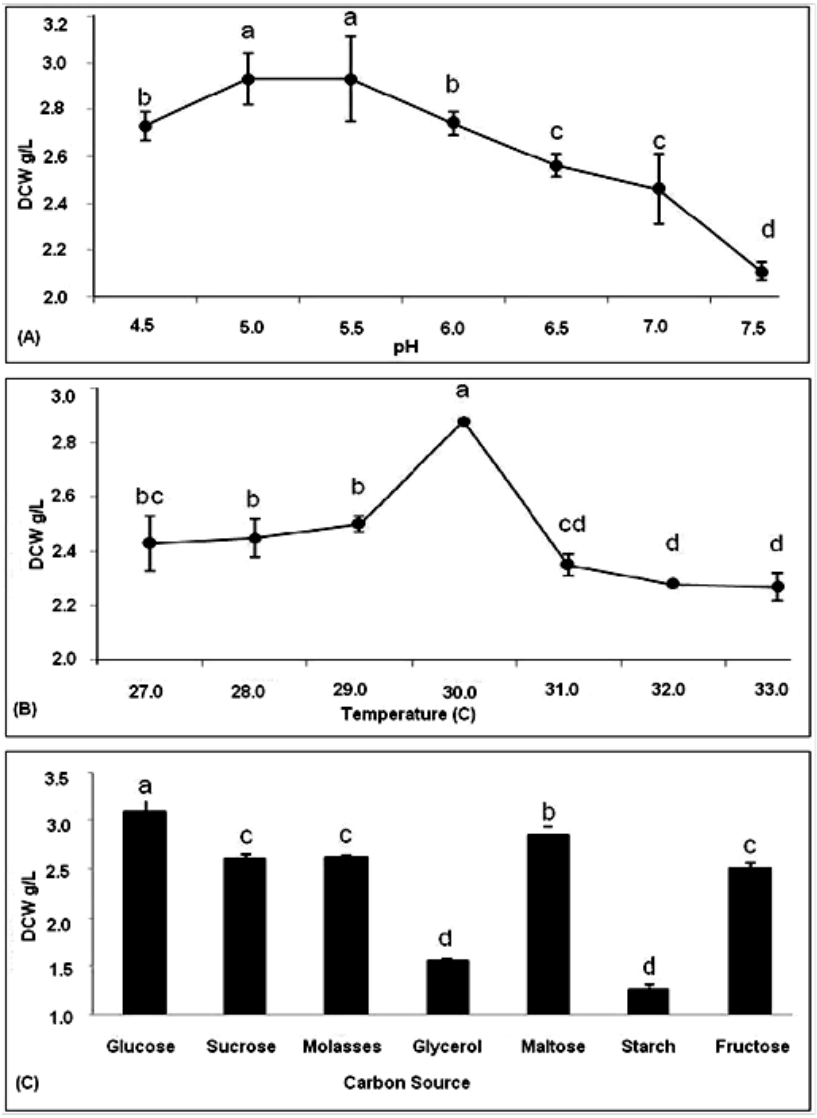

Figure 1. Comparison of yeast dry cell weight (DCW) under different pH (A), temperature (B), and carbon source (C). Error bars indicate ± SE. Points/bars bearing different letters are significantly different according to DMRT ($p<0.05$).

Table 2. Comparison of dry cell weight and Se uptake in different media in bio reactor.

Media	Dry cell weight (g/L)	Se in yeast biomass (ppm)	MBRT
Defined media - A	9.6 ± 0.22^{d}	1980.0 ± 14.70^{d}	< 10 min
Defined media - B	10.5 ± 0.14^{c}	2251.3 ± 10.36^{c}	< 5 min
Synthetic media	12.8 ± 0.22^{a}	2718.3 ± 6.24^{a}	> 15 min
Rich media	11.7 ± 0.36^{b}	1640.0 ± 10.80^{e}	< 1 min
Industrial media	$10.2 \pm c\ 0.29^{c}$	2457.7 ± 6.13^{b}	< 10min

*Mean ± SE. Means in a column followed by different superscripts are statistically different according to DMRT ($p<0.05$).

Moderate Se uptake and biomass yield was obtained both in defined media A and B.

The methelene blue reduction time was less than 1 min for rich media which indicates the presence of the highest

inorganic Se content. The defined media-B had the reduction time of less than 5 min, indicating the presence of a level of inorganic Se. The reduction time was upto 10 min for the defined media-A and industrial media, indicating the presence of traces of inorganic Se. The MBRT was more than 15 min for yeast grown on synthetic media. This strongly implies the presence of only traces of inorganic Se in the medium and hence demonstrate the highest organic Se accumulation (Table 2).

The results clearly indicate that the synthetic medium containing less sulfur supported maximum biomass and the highest organic Se accumulation. The maximum concentration (2718 ppm) of Se incorporated into the yeast cells in the present study was in the synthetic medium. This minimum Se incorporation observed was only 10% less compared to the highest amount of Se (3000 ppm) that could be incorporated into yeast cells (Schrauzer, 2006). The values for MBRT method for the analysis of extra cellular inorganic Se was also very high in the case of synthetic medium indicating the highest accumulation of organic Se in yeast cells.

Conclusion

From this study, we optimized the culture medium and culture conditions for the production of high cell densities of yeast enriched with high Se content. Maximum biomass yield in shake flask experiment was achieved under an optimum pH 5 and at an optimum temperature of 30°C. We also found that glucose is the best and simple carbon source which facilitates the rapid proliferation and high Se incorporation into yeast cells. The synthetic medium, containing amino acid mixture, vitamins and trace minerals, without any complex nutrients like peptone and yeast extract supported maximum yeast cell proliferation (12.8 ± 0.22 g/L), Se accumulation (2718 ± 6.24 ppm) and highest MBRT (>15 m) which may be due to less sulfur content of the medium (Ouerdane and Mester, 2008). However, industrial media recorded the second highest organic Se accumulation, even though it contains the complex nutrients like molasses, corn steep liquor and yeast extract. From this study, we concluded that industrial media can be used for the large scale production of Se yeast as this also supports high Se accumulation in yeast biomass and is also very economical.

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