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Selenium supplementation during fermentation with sugar beet molasses and *Saccharomyces cerevisiae* to increase bioethanol production

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Abstract: A bench scale submerged fermentation process was used to bioethanol produce using sugar beet molasses and *Saccharomyces cerevisiae*, as substrate and microbial strain, respectively. Effects of selenium amount on growth of *S. cerevisiae* and bioethanol production were evaluated. The obtained results indicated that growth of *S. cerevisiae* (manifested as turbidity intensity) in the samples containing 0, 5, 10, 15, 20 and 25 µg sodium selenite, during aerobic process, was 0.1707, 0.1678, 0.1679, 0.1664, 0.1627 and 0.160% a.u./h (after 14 h incubation), respectively. Statistical analysis based on compression test indicated that there were insignificant ($p > 0.05$) differences between growth rate of the yeast in the fermented samples containing *S. cerevisiae* and 5 to 25 µg selenium salt. Response surface methodology was utilized to evaluate effects of two fermentation parameters namely, amount of selenium (5-25 µg) and substrate brix (10-25°Bx) on the concentration (g/L) of produced bioethanol. Obtained results revealed that maximum bioethanol concentration (55 g/L) was achieved using 15 µg selenium and molasses with 25°Bx. Furthermore, results have also indicated that, without using selenium and using molasses with 25°Bx, bioethanol with concentration of 29 g/L was produced.

Keywords: bioethanol overproduction; *Saccharomyces cerevisiae*; selenium; substrate brix; optical density

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1 Introduction

Food wastes contain main carbohydrates such as pectic, starchy and sugary compounds which those are accumulated every year and caused ecological problems. Incorporation of biotechnology methods and approaches into chemical and environmental engineering aspects, is of great interest to overcome the environmentally concerns resulted by the wastes. Recently, food and agro-wastes have gained more attention to be utilized in biotechnologically processes as an enriched and suitable substrate to produce valuable products such as food additives, enzymes, antibiotics, organic acids, biofuel and biogas [1].

Bioethanol is known as clean, cost-effective and eco-friendly fuel and has been widely utilized in developed countries as alternative and replacement of the fossil fuels [2,3]. In fact, bioethanol can also be used as gasoline improver or octane enhancer to increase flames speed and heats of vaporization with minimum toxicity and air-borne pollutants [4]. Therefore, its production through submerged fermentation and using agro-industrial by-products, especially sugar beet molasses, is an attractive and eco-friendly topic these days.

Several studies have been indicated that the yield of bioethanol production through fermentation, influences by numerous parameters such as type and volume of inoculum (microorganisms strain), composition and concentration of substrate (growth media), pH, temperature, osmotic pressure of the culture media, and presence of nutrients, minerals and precursors [5,6]. Selenium as an essential trace element has crucial role in animal lives, human health and micro/macro flora. But, due to its low concentration in vegetables (as human diets) and its short bioavailability, many people are poor because of this vital element [7,8]. Conversion of selenium to organic selenium compounds such as seleno-proteins (especially seleno-methionine and seleno-cysteine) and seleno-enzymes, improves selenium deficiency in the human body and shows numerous biological activities such as antioxidant and anti-inflammatory activities

[8,9]. Several studies indicated that yeast cells are capable to absorb selenium and bio-transform it into seleno-methionine and seleno-cysteine [10-13]. Selenium-enriched yeasts, which are known as selenised yeasts, are the base of many consumed supplements and most popular. For examples, enriched yeasts with selenium, such as *Saccharomyces cerevisiae*, *S. bayanus* and *S. boulardii* have been utilized to produce bread, probiotic products and alcoholic beverages [12,14,15]. Several studies indicated that, glutamate in the yeast, can decrease the energy generation and fermentation rate, by altering the mitochondrial structure and dynamics, which those negatively impacts can be prevented by organic selenium such as seleno-cysteine which that is resulted during bio-transformation of inorganic selenium using yeasts such as *S. cerevisiae* [12,14]. Other study indicated that contents of organic selenium in the *S. cerevisiae* during fermentation process to produce bioethanol, increased with the increase in selenium concentration up to 2 µg/mL followed by a gradual decrease after 24 h of incubation. It reveals that organic selenium has low bioavailability [16]. Furthermore, some species of *Saccharomyces*, such as *Yarrowia lipolytica*, has selenium tolerance and selenium in lower amounts, cannot significantly inhibit its growth [15]. To the best of our knowledge, there is not any comprehensive study to evaluate effects of selenium salt and culture media concentrations on production of bioethanol through submerged fermentation process using *S. cerevisiae* and sugar beet molasses. Furthermore, the mechanism of the selenium on production of bioethanol using *S. cerevisiae* is unknown.

Therefore, the main objectives of the present study were to i) evaluate the effect of selenium on the concentration of produced bioethanol, ii) optimize submerged fermentation parameters namely substrate and selenium concentrations to achieve bioethanol with highest concentration, and iii) compare concentration of produced bioethanol, between cultivation made with and without selenium.

2 Material and methods

2.1 Materials

Sugar beet molasses, as substrate, was provided from Sahand Company (Khoy, Iran). It has °Brix, total reduced sugar amount, pH, ash content and density values of 74.07 (°Bx), 48.65%, 6.16, 9.6 (% v/v) and 1.385×10^3 (kg/m^3), respectively. Commercial *S. cerevisiae* strain, SFO6, was obtained from Iran Mayeh Company (Tehran, Iran). 30% v/v sulfuric acid (as pH adjuster), Diammonium hydrogen phosphate (as phosphorus source) and urea (as nitrogen supplement) were purchased from the Dr. Mojallali Company (Tehran, Iran). Sodium selenite (Na_2SeO_3) with purity of higher than 99% (as selenium precursor) was provided from Merck Company (Merck Co., Darmstadt, Germany).

2.2 Inoculum preparation through aerobic process

Provided molasses was sterilized using a laboratory autoclave (RT-1, Reyhan Teb, Tehran, Iran), adjusted at temperature of 121°C and pressure of 1.5 bar for 15 min, diluted using sterilized distilled water to prepared aqueous molasses with brix value of 11 (°Bx) using a refractometer (Index instrument Ltd., Kissimmee, FL, USA), enriched with 250 mg/L urea and 500 mg/L urea and diamonium hidrogen phosphate, and adjusted its pH to 4.2. After that, prepared substrate containing 0.3 g/L provided industrial yeast, was aerated using shaker incubator (S1-300, Jeio Tech, Daejeon, Korea,) 120 rpm, 1 (VVM) at 32°C for 24 h.

2.3 Growth of *S. cerevisiae* in the prepared molasses

Optical density measurement was used to monitore the growth of *S. cerevisiae* in the provided molasses with °Bx of 11, as mentioned in inoculum preparation through aerobic process. In order to evaluate of the effect of selenium amounts (0, 5, 10, 15, 20 and 25 µg) on the growth of the *S. cerevisiae*, after preparation of molasses containing 0.3 g/L of the yeast, defined amounts of selenium were added into that and the absorbance of the prepared samples, as an optical density, was measured every 1 h, using UV-Vis spectrophotometry (Jenway UV-Vis spectrophotometer 6705, Staffordshire, UK) adjusted at wavelength of 625 nm [5,6]. For this reason, samples were diluted 4 times with distilled water to decrease their colour intensity and after that, those were subjected to the spectrophotometer. The recorded values were then multiplied in 4 to obtain exact value of optical density for the samples.

2.4 Bioethanol production through anaerobic submerged fermentation and its concentration measurment

Bioethanol was produced using anaerobic batch submerged fermentation when, 60 mL of the prepared

inoculum with 11°Bx and different amount of sodium selenite (5-25 µg) were added into the 140 mL of the diluted and sterilized molasses with different brix value ranging 10 to 25°Bx. The mixture solutions were then filled into the 250 mL rubber sealed glass jars and incubated (at 32°C for 32 h). Finaly, the concentration of the produced bioethanol was calculated using technique based on distillation. In this manner, after bioethanol distillation and true brix measurement in distilled fermentation broth by a refractometer (Index instrument Ltd., Kissimmee, FL, USA), bioethanol concentration was measured using a hydrometer [5].

2.5 Experimental design and statistical analysis

According to the literature studies, two independent variables namely, amount of sodium selenite (µg, X_1) and substrate brix (°Bx, X_2) were selected to evaluate their effects on bioethanol concentration (g/L, Y), as response variable, using response surface methodology (RSM) [6,8,9,14]. Central composite design (CCD) was utilized to design of experiments, including 13 experiment runs (Table 1), based on axial point system and 1 block [17,18]. In order to model the bioethanol concentration (g/L, Y) as function of two selected independent variables, a second order polynomial equation was selected [19,20]. Suitability of the generated model was studied based on the coefficient of determination (R^2) and lack-of-fit p-value [21,22]. In order to significance determination of the resulted model, analysis of variance (ANOVA) was used based on p-value term ($p < 0.05$) [23,24]. Minitab software (v.16 statistical package, Minitab Inc., PA, USA) was used to design of experiments and statistical analysis.

2.6 Optimization of the bioethanol fermentation conditions

To find optimum area with in defined ranges for the fermentation variables, contour plot was establish [25]. Furthermore, to obtain the exact values of the optimized fermentation conditions which in that bioethanol with highest concentration were produced, numerical optimization was used [6]. Three additional approval tests were performed at obtained optimum conditions to verify the validity of the statistical experimental method [26]. For this reason, Tukey's comparison test was performed between the values of the predicted and experimental bioethanol concentration at obtained optimum fermentation conditions. Minitab software (v.16 statistical package, Minitab Inc., PA, USA) was used to optimization and validation procedures.

Table 1: Central composite design (CCD) for the bioethanol production using *S. cerevisiae*.

Run	Selenium amount (µg)	Substrate brix (°Bx)	Experimental bioethanol concentration (g/L)	Predicted bioethanol concentration (g/L)
1	15	10	15	15.04
2	15	25	55	55.20
3	5	17.5	27	27.38
4	8	12.2	17	16.74
5	15	17.5	29	29.00
6	15	17.5	29	29.00
7	15	17.5	29	29.00
8	25	17.5	33	32.86
9	22	22.8	*	*
10	8	22.8	45	44.63
11	15	17.5	29	29.00
12	15	17.5	29	29.00
13	22	12.2	20	20.11

* Out of range

3 Results and discussion

3.1 Effects of selenium on the growth of *S. cerevisiae*

Effects of different amounts of selenium on the growth (manifested as turbidity intensity) of *S. cerevisiae*, show in Figure 1. As clearly observed in this figure, the growth of the yeast in the provided molasses without selenium was significantly ($p < 0.05$) higher than that of those which were included with different amounts of selenium. According to the Figure 1, the slope of each curve, as growth rate, was calculated from beginning of the experiments up to 14 h after incubation. The obtained results indicated that growth rate (manifested as turbidity intensity (% a.u.)) of the *S. cerevisiae* in the samples including, 0, 5, 10, 15, 20 and 25 µg selenium was 0.1707, 0.1678, 0.1679, 0.1664, 0.1627 and 0.160% a.u./h, respectively. Tukey's comparison test was indicated that there was insignificant ($p > 0.05$) differences between growth rate (manifested as turbidity intensity) of the *S. cerevisiae* in the samples containing *S. cerevisiae* and 5 to 25 µg selenium. It can

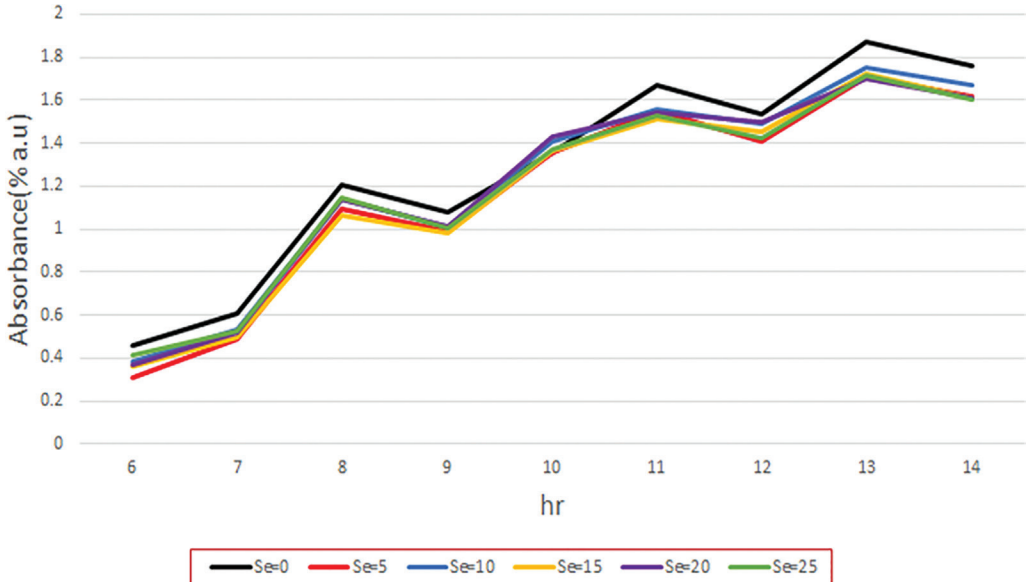

Figure 1 Effects of different amounts of selenium on the growth of *S. cerevisiae*.

be related to the lower bioavailability of the organic selenium in *S. cerevisiae* [16]. The obtained result was also in agreement with findings of Hamza et al. [15]. They also found that some species of *Saccharomyces*, had selenium tolerance and selenium in lower amounts, could not be significantly inhibit its growth.

3.2 Effects of substrate brix and selenium amount on the concentration of produced bioethanol

According to the experiment runs and obtained values for the concentration of produced bioethanol through submerged fermentation (Table 1), the second order model fitted to correlate bioethanol concentration to the fermentation parameters, namely selenium amount and inoculum brix. Estimated regression coefficients and P-values of the main, quadratic and interaction terms of the generated polynomial model are presented in Table 2. As clearly observed in Table 2, the main term of the selenium amount and its interaction with medium brix had insignificant ($p > 0.05$) effects on the concentration of bioethanol. But, the main term of medium brix and quadratic terms of both selected fermentation parameters had significant ($p <> 0.05$) effects on the produced bioethanol concentration. It means that with in the defined ranges for the independent variables, concentration of the produced bioethanol affected by lower and higher brix of the medium, and only higher amounts of selenium salt. Statistical analysis had also shown high values

Table 2: P values and regression coefficients for the generated model using *S. cerevisiae*.

P-value			Regression coefficient	
Parameters	Independent variables	P-value	β	Coefficient
Constant		0.000	β_0 (Constant)	15.71
Main term	X_1	0.135	β_1	- 0.18
	X_2	0.000	β_2	- 1.23
Quadratic term	X^2_1	0.002	β_{11}	0.01
	X^2_2	0.000	β_{22}	0.1
Interaction term	X_1X_2	0.189	β_{12}	0.00
R^2		0.9997		
Lack-of-fit (p-value)		0.420		

1: Amount of selenium (µg)
2: Substrate brix (°Bx)

for the R^2 (0.9997) and lack-of-fit (p-value of 0.420) of the generated model which those indicated suitability and accuracy of the resulted model for predicting of bioethanol concentration with in the defined ranges for the fermentation parameters [19,22].

As can be seen in Table 1, the concentration of the produced bioethanol was varied from 15 to 55 g/L. Figure 2, indicates the effects of selenium amount and substrate brix on the concentration of produced bioethanol. As can be seen in Figure 2, at any constant

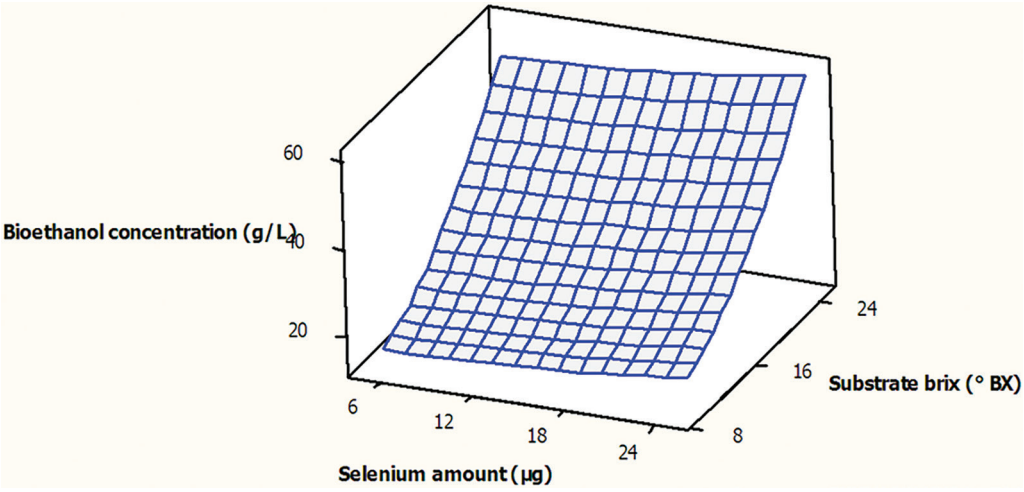

Figure 2: Surface plot for concentration of the produced bioethanol (g/L) as function of the substrate brix (°Bx) and amount of selenium (µg), during submerged fermentation.

amount of selenium, by increasing the brix of substrate, bioethanol concentration, increased. This result was in agreement with finding of Shaghaghi-Moghadam et al. [6]. They indicated by increasing the substrate brix, the concentration of the fermentable sugar increased which in turn, increased the concentration of the produced bioethanol. One of the factors which can highly affect the fermentation performance is the medium osmotic pressure which that increases by increasing the medium brix and may negatively affect the yeast growth and bioethanol production [6]. As same as this pattern, at any constant substrate brix, by increasing the amount of selenium, the concentration of the produced bioethanol was increased. However, the effect of substrate brix on the increasing of the bioethanol was higher than the selenium amount, due to its lower p-value (0.000). The presence of no curvature in the Figure 2 also demonstrated that the interaction between substrate brix and amount of selenium did not has significant ($p < 0.05$) effect on bioethanol concentration. Obtained result was reconfirmed by achieved high p-value ($p > 0.05$) of the interaction term (0.189) as can be observed clearly in Table 2.

3.3 Optimization of the fermentation process

In order to achieve bioethanol with highest concentration through submerged fermentation, the obtained numerical optimization result revealed that fermentation using 15 µg sodium selenite and substrate with 25°Bx attained to produce bioethanol with highest concentration value of 55.2 g/L. Graphical optimization shows in Figure 3. As can

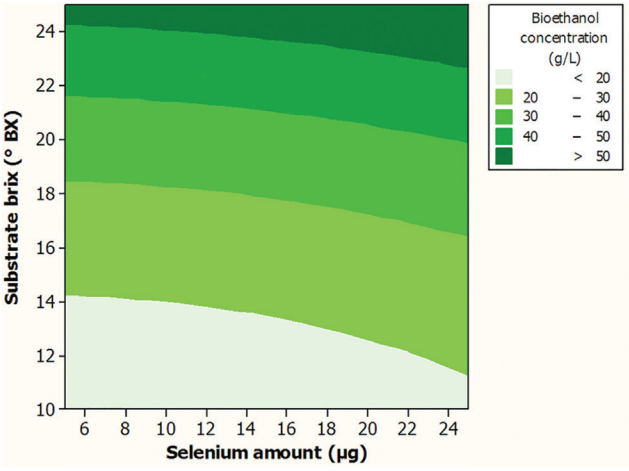

Figure 3: Graphical optimization plot for concentration of the produced bioethanol (g/L) as function of the substrate brix (°Bx) and amount of selenium (µg), during submerged fermentation.

be seen in this figure, at any constant substrate brix value, for lower amount of selenium, by increasing the amount of selenium, the concentration of produced bioethanol was constant and at higher amounts of selenium, increasing its amount had significant ($p < 0.05$) effect on the concentration of bioethanol. This result was reconfirmed by the obtained statistical data for the insignificant effect of lower amounts of selenium (p-value = 0.135) on the concentration produced bioethanol (Table 2). Graphical optimization plot was illustrated that, with in the defined ranges for the independent variables, maximum bioethanol was achieved using highest substrate brix values. Experimental data for the obtained bioethanol concentration (55 ± 2 g/L) using the optimum fermentation parameters revealed that there was insignificant ($p > 0.05$)

difference between the values of the experimental and predicted concentration of produced bioethanol and indicated the adequacy of the fitted model.

In this work, selenium was applied in a submerged fermentation to study its effects on production of bioethanol. For this reason, an anaerobic fermentation process was run at same conditions as obtained optimum conditions without selenium. In fact, at this fermentation process, the substrate brix was chosen at 25°Bx and the amount of sodium selenite was zero. Obtained result indicated that the concentration of produced bioethanol was 29 g/L which was 52.3% lower than that of obtained using 15 µg selenium (55 ± 2 g/L). Obtained result can be explained by the fact that existed glutamate in the yeast, negatively impacts the mitochondria by altering the mitochondrial structure and dysregulation of mitochondria dynamics which is decreased the energy generation and fermentation rate. *S. cerevisiae* by converting inorganic selenium to seleno-cysteine, utilized this seleno-amino acid to prevent glutamate-induced effect [27]. It seems that *S. cerevisiae* has high potential to bio-transform selenium to organic selenium compounds such as seleno-proteins which was confirmed by findings of Pérez-Corona et al. [14] and Porto et al. [12].

4 Conclusions

Present study indicated that production of bioethanol with high concentration, through submerged fermentation process, could be resulted by increasing the concentration of molasses (substrate) and utilizing high amounts of selenium, as regulator of the yeast mitochondria dynamics function. The obtained results indicated that lower amounts of selenium had insignificant effect on the concentration of the produced bioethanol. Results also revealed that the selected industrial *S. cerevisiae* strain had high resistance against osmotic pressure of the fermented broth which it makes possible to achieve bioethanol with twice concentration when comparison was made between cultivation made with and without selenium using highest substrate brix. Finally, RSM could be successfully used to generate model, optimize the process and predict the bioethanol concentration with in the defined ranges for the selenium amount and substrate brix.

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Conflicts of interest: All authors declare no conflict of interest.

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