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Production of Zn-Enriched Yeast

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Abstract: Zinc (Zn) enriched *Saccharomyces cerevisiae*, was produced as a source of Zn. The impact of process variables on the bioaccumulation of Zn in yeast was studied by Plackett-Burmann and Box Behnken designs. For the first time impact of ultrasounds was investigated as a tool to stimulate Zn accumulation. The optimum growth condition was reported in which total Zn accumulation jumped by twice compared to similar conditions without ultrasounds stimulation. The results of this study revealed a suitable solution to overcome the deficiency problem of Zn is the production of Zn-yeast.

Keywords: yeast; design of experiments; Plackett-Burmann design; Box-Behnken; ultrasound; bioaccumulation enrichment; Zn.

1. Introduction

Since Zinc essentiality for plants, experimental animals, and humans was established in 1869, 1934, and 1961, zinc deficiency in the human diet has remained a critical problem. So that the World Health Organization introduced zinc deficiency as the 11th highest risk factor for disease mortality and morbidity in 2002, while the matter is zinc bioavailability for living organisms [1], e.g., chicken [2, 3]. Catalysis of several biochemical reactions in living organisms depends on various organic and inorganic factors, such as enzymes, metalloproteins, and minerals, such as zinc, magnesium, selenium, etc. [4]. In addition to the indispensable role of zinc in different types of metalloenzymes as a cofactor, this element contributes significantly to the structure and synthesis of negatively charged macromolecules like DNA double-helix and proteins. Besides, this element can decrease free radicals generation and prevent apoptotic cell death [5]. The most bioavailable forms of zinc and selenium for humans are organic compounds of Zinc and Selenium, and we can name Zn^{2+} and/or Se^{4+} enriched biomass as one of the most efficient sources to supply these microelements [6, 7].

Metals can be accumulated via microorganisms through various processes, including biosorption, bioprecipitation, transportation, oxidation-reduction reactions, and entrapment in extracellular capsules [8]. By and large, these processes are defined in two major categories of passive capture and active capture. The first stage, termed "biosorption", is the process of cation accumulation on the outer surface of a cell wall, followed by metal adsorption on anionic binding sites of the microbial cell wall [9]. While the second stage, known as "bioaccumulation", is an intracellular and metabolism-dependent uptake, processed by specific

transporters located on/in the cell membrane, which are able to enter the metal ions to the cell and its metabolic cycles [7, 8].

Among a variety of living organisms, Saccharomyces cerevisiae could be one of the best sources of biomass due to its cost-effective price of production, being easily available. Moreover, this organism's ability to accumulate cationic metals such as Co^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Pb^{2+} has been proven during recent decades [7, 10-17].

Ultrasonication has been recently used as an auxiliary method for fermentation enhancement. This method could be applied directly, via an ultrasonic probe, or indirectly using an ultrasonic bath and is now applicable for a variety of processes in biotechnology like fermentation, extraction, and different biological reaction catalysis. Besides, we can see this method's application in the food industry, pharmaceutical production, and waste disposal [18, 19].

Ultrasonic waves frequency are considered to be above 18 KHz. While ultrasonic waves above 100 KHz, as known as high-frequency waves, are famous for their application in cell disruption, we can use low-frequency waves below 100 KHz to increase the productivity of microbial cells [20].

The mechanism of this enhancement has a long way to go to be completely clear. However, we know this is a result of increasing mass transfer between the inside and outside of the cells. When an ultrasonic probe is applied in a liquid media, it will cause alternating compression generation and rarefaction cycles which makes a rapid reduction in the pressure of the system and results in bubble generation that is followed by their expansion and implosion. This phenomenon, named cativation, causes an increase in the temperature and pressure of the system. This temperature enhancement is usually controlled using recirculating cooling water to treat microorganisms in process temperatures under 40 ˚C [19-21]. Consequently, there will be an increase in cell membrane permeability, calcium channel activation, higher transportation of preferable nutrients, and finally, mass transfer enhancement between the inside of the cells and outside [19, 22].

2. Materials and Methods

2.1. Organisms and media preparation.

Saccharomyces cerevisiae (ATCC 9763) was purchased from the culture collection of the Iranian Research Organization for Science and Technology (IROST).

Sabouraud dextrose agar (SDA) (Merck, Germany) was used to maintain and isolate colonies. A YPD medium was prepared by combining the following ingredients (g/l): (3) yeast extract, (5) peptone, and (10) glucose [23].

1 liter of basic culture medium consisted of deionized water (671.60 ml), date molasses (246.7 ml), NH_4Cl (7.5 g), $MgCl_2$ (0.3 g), KH_2PO_4 (2.5 g), different concentrations of Zinc chloride suspension (600 µg/ml, 1300 µg/ml or 2000 µg/ml) and different concentrations of Selenium suspension (45 µg/ml, 30 µg/ml or 60 µg/ml), calcium pantothenate (0.025 g), thiamin (0.2 g), sodium citrate (15 g) at pH 5.5 ± 0.1 [24].

All the media were autoclaved at 120 ˚C under a pressure of 1.4 atm for 20 minutes for sterilization after preparation.

2.2. Inoculum preparation and cultivation.

To cultivate the yeast species, *Saccharomyces cerevisiae* was passaged on Sabouraud dextrose agar (SDA) (Merck, Germany) and incubated at 30 ˚C in the dark. After 48 h, two colonies were transformed to 100 ml of sterile YPD medium and incubated at 30 ˚C while shaking at 180 rpm, and inoculum was prepared after 24 h [22]. For the next step, 10% (v/v) of cultivated YPD medium was inoculated to the basic culture medium with a final volume of 100 ml. The fermentation was followed at 30 ˚C in a shaking incubator at 180 rpm for 24 h.

2.3. Ultra-Sonic treatment.

For each run, after 10 h, when the population of yeasts in the fermented medium was in the exponential phase of growth, the ultra-sonic treatment got started. In this study, a probe sonicator was used. The probe was placed into an Erlenmeyer of 250 mL capacity, containing 100 mL fermented media, and the process continued at 30 ˚C, using Ice packs to cool down the temperature. The liquid culture was irritated with a power density of 140 W/L, frequency of 20 kHz, and 20% duty cycle (pulse duration of on-time 2 s and off-time 8 s) in three different sonication times of 180 minutes, 135 minutes, and 90 minutes [25]. After ultra-sound treatment, the flask was returned to the shaking incubator, and the fermentation followed under the previously determined condition.

2.4. Design of experiments.

2.4.1. Plackett-Burman Design (PBD).

The variables to be evaluated were listed based on the literature review and pre-experiences. Table 1 shows the variables and two levels used in this study's screening method of Plackett-Burmann design. Regarding our screening step, analyzing factors impacting Zn absorption by yeast, three independent variables were chosen by Box Behnken design for the next research step. The main variables include Zn concentration, Se concentration, and the time span of exposing culture media to ultra-sonic waves. According to the principles of Box-Behnken design, these factors were taken as variables tested in a 17-run experiment design to determine the optimized condition (Table 2).

3. Results and Discussion

3.1. PBD.

Fifteen variables were analyzed to evaluate their impact on the accumulation of Se using a PBD (Table 1). To find cost-effective fermentation conditions for SeY production by *S. cerevisiae*, four significant variables were selected by PBD and optimized using BBD. The variables coefficient is exhibited in Figure 1. After screening 15 dependent variables in SeY production, 4 effective factors with higher impact coefficients, including the degree of brix, Se concentration, ultrasound power, and duty cycle, were selected to optimization of effective process variables by Box Behnken design.

The results represented that three variables significantly impacted Zn accumulation (Zn and Se concentration and ultrasound duty), whereas the other variables (Mg concentration, power, and time of treatment) increased Zn bioaccumulation.

Table 1. Plackett Burman Design for evaluation of 15 process variables on Se enriched yeast.

No	Carbon source	Brix of carbon source	Nitrogen source	Nitrogen concentration (g/l)	Se concentration (µg/ml)	Zn concentration (µg/ml)	Mg concentration (g/l)	K concentration (g/l)	Time of incubation (h)	Shaking (rpm)	Ultrasound duration (h)	Growth phase for treatment	Power of ultrasound (watt)	Inoculum treated with ultrasound	Duty cycle (%)	Dried Cell Weight (g/L)	Organic Se (mg/L)	
																	Predicted	Observed
1	1	20	0	7.5	40	100	0.3	2.5	48	130	2.5	0	140	1	60	10.8	164.8	164.8
2	1	45	0	7.5	15	1000	0.3	0	48	180	0.5	1	70	1	60	1.4	23.7	0.0
3	1	45	1	7.5	15	100	2	0	24	180	2.5	0	140	0	60	0.6	0.0	0.0
4	1	45	1	15	15	100	0.3	2.5	24	130	2.5	1	70	1	20	0.5	0.0	0.0
5	0	45	1	15	40	100	0.3	0	48	130	0.5	1	140	0	60	9.8	91.4	91.4
6	1	20	1	15	40	1000	0.3	0	24	180	0.5	0	140	1	20	11.9	136.3	139.4
7	0	45	0	15	40	1000	2	0	24	130	2.5	0	70	1	60	7.6	182.8	182.8
8	1	20	1	7.5	40	1000	2	2.5	24	130	0.5	1	70	0	60	9.2	210.8	210.8
9	1	45	0	15	15	1000	2	2.5	48	130	0.5	0	140	0	20	0.6	0.0	0.0
10	0	45	1	7.5	40	100	2	2.5	48	180	0.5	0	70	1	20	8.6	30.5	123.2
11	0	20	1	15	15	1000	0.3	2.5	48	180	2.5	0	70	0	60	11.4	42.8	42.8
12	1	20	0	15	40	100	2	0	48	180	2.5	1	70	0	20	10.1	154.8	154.8
13	0	45	0	7.5	40	1000	0.3	2.5	24	180	2.5	1	140	0	20	10.2	125.2	125.2
14	0	20	1	7.5	15	1000	2	0	48	130	2.5	1	140	1	20	12.3	32.4	35.5
15	0	20	0	15	15	100	2	2.5	24	180	0.5	1	140	1	60	6.2	25.9	25.9
16	0	20	0	7.5	15	100	0.3	0	24	130	0.5	0	70	0	20	11.2	21.6	21.6

Table 2. Box-Behnken design with experimental levels of variables.

Run	Factor 1	Factor 2	Factor 3							
	A: Selenium concentration (µg/ml)	B: Zinc concentration (µg/ml)	C: Ultra-Sonic treatment duration (min)	Dry biomass cell weigh(gr)	Total Zn absorbance (ppm)	Inorganic Zn absorbance (ppm)	Total Se absorbance (ppm)	inorganic Se absorbance (ppm)	organic Zn absorbance (ppm)	Organic Se absorbance (ppm)
1	45	1300	135	0.0782	36.69	19.55	28.73	0.40	17.14	28.33
2	60	2000	135	0.0973	41.80	5.52	98.73	0.42	36.28	98.31
3	45	1300	135	0.0880	12.51	1.74	37.38	0.51	10.77	36.87
4	30	2000	135	0.0885	24.17	2.06	22.50	0.46	22.11	22.04
5	45	2000	180	0.0959	25.71	2.73	35.33	0.46	22.98	34.87
6	45	2000	90	0.0918	27.35	4.59	58.47	0.50	22.76	57.97
7	30	1300	90	0.0726	14.59	0.85	20.13	0.44	13.74	19.69
8	45	1300	135	0.0523	7.22	0.67	30.02	0.48	6.55	29.54
9	45	1300	135	0.0860	12.71	1.49	41.21	0.50	11.22	40.71
10	45	1300	135	0.0836	9.91	0.89	49.92	0.59	9.02	48.63
11	60	600	135	0.0836	5.14	0.54	59.43	0.52	13.62	58.91
12	60	1300	180	0.0733	9.19	1.03	61.42	0.84	8.16	60.58
13	30	600	135	0.0811	6.08	1.47	31.56	0.71	4.61	30.85
14	60	1300	90	0.0816	8.13	0.85	75.20	0.72	7.28	74.48
15	30	1300	180	0.0841	28.14	1.04	15.09	0.56	27.1	14.53
16	45	600	180	0.0785	5.50	0.74	62.90	0.72	4.76	62.18
17	45	600	90	0.0927	35.67	1.82	63.20	0.55	33.85	62.65

3.2. Cell Biomass yield determination.

To measure yeast cell biomass yield, 10 ml of the fermented liquid was transformed into pellets and then centrifuged at 4000 ×g at 4 ˚C for 10 minutes. After decanting the supernatant, the process of washing with deionized water and centrifuging was repeated two times. The pellets were heated at 80 ˚C to reach a constant weight. The biomass weight was determined after cooling [26].

3.3. Determination of Zn bioabsorption.

To determine Zn absorption by the yeast cells, 35 ml of the fermented liquid were centrifuged at 3500 ×g for 10 minutes at 25 ˚C, and the process was repeated 3 times. After freeze-drying, 100 mg of dried biomass was exposed to 3 ml HNO3 65% and heated at 105 ˚C for 20 minutes using a heat bath. After cooling, 1 ml HCl 37% was added to the pellets and heated at 80 ˚C for 10 minutes. The total Zn concentration was then measured by the ICP-OES system. To determine inorganic Zn, 100 mg of dried biomass was suspended in 5 ml of deionized water and boiled in a heat bath for 60 minutes. After cooling, the liquid was centrifuged at 8300 ×g for 15 minutes. The amount of inorganic Zn in the supernatant was determined using ICP-OES. The organic Zn concentration would be reached by subtracting the amount of inorganic Zn from the total Zn [24, 27].

In a study, in order to obtain an optimum level of Zinc-yeast production, Zinc sulfate was used as the source of zinc with various concentrations of 200, 300, and 400 mg/L, and the fermentation process continued for 84 hours before adding the Zinc source and 24 hours after that [13]. However, in the present study, we used different concentrations of 600 µg/ml, 1300 µg/ml, and 2000 µg/ml of Zinc chloride, and we could hasten the fermentation process to 48 h using different treatments with Ultrasonic waves which could speed up the Zn uptake by yeast cells.

In another study, It has been reported that after testing different concentrations of zinc sulfate, zinc chloride, and zinc nitrate as Zinc sources, the highest uptake was achieved when Zinc sulfate was used at a concentration of 120 mg 100 ml−1. Besides, they have reported that after 30 ppm, the higher concentration of Zn is used, the lower biomass yield is reached, and in this study, this phenomenon has been attributed to the harmful impact of Zn on yeast cells when it is available in the culture medium, more than it is necessary for the cells to be used in the regulation of cell structure and metabolic activity [10].

There is another study in which Zn-enriched yeast was produced under different conditions, aiming to enhance bakery products and extend shelf-life by testing Zn-yeast Anti-mycotoxigenic properties. In this study, different Zinc salts, a range of pH, different intact times, zinc sulfate concentrations, and different temperatures were selected as variables to reach the optimum condition of Zn uptake by yeast cells [12]. While in the present study, we tried to reach the optimum condition, testing the variables of Zn concentration, Ultrasonic treatment period, and Se concentration altogether.

Moreover, it is reported that producing ZnYeast cells using ZnSO4·7H2O as the source of Zinc *Saccharomyces pastorianus Rh* as the yeast cell could make the intestinal zinc bioavailability comparable to other organic and inorganic Zinc sources after digestion of produced yeast biomass in an invitro model. Similar to the present study, this research has introduced ZnYeast as a suitable nutritional source for defeating Zn deficiency [11].

While we used ultrasonic waves as a tool to enhance Zn uptake, in another study, an attempt has been made to enhance Zn and Cobalt uptake using a siderophore-containing medium. They have evaluated the role of different siderophores obtained from different microorganisms in Zn and Copper transportation to yeast cells [12].

Regarding the tremendous impact of Zn on human health, it seems that using ultrasonic temperature could be not so difficult for the enrichment of yeast (as an additive of bread, bakery and brewing products) and its application in food [28-31].

4. Conclusions

The present study used PBD to test the relative importance of culture conditions and process variables on cell growth and the production of Zn-enriched yeast. Screening of the variables influencing Se biotransformation by yeast, including duration and power of ultrasound, inoculum treatment with ultrasound, duty cycle, growth phase, time, shaking rate, inorganic salts concentration (Se, Zn, Mg, and K), nitrogen and carbon sources as well as their concentrations by using Plackett–Burman design. The lack of fit was insignificant ($P>0.01$). The optimum condition for Se accumulation was obtained at Zn and Se concentration of 80 and 60 µg/ml, ultrasound of 90 W/L, and duty cycle 40%. Using various fermentation conditions and ultrasounds, Zn accumulation increased from 138.9 mg/ kg to 290.2 mg/kg.

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Conflicts of Interest

The authors declare no conflict of interest.

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