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Optimization of culture conditions of iron-enriched biomass of *Saccharomyces pastorianus* by response surface methodology

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Abstract:

Yeast biomass enriched with iron is used in a profound and safe treatment for anaemia. In this work, response surface methodology (RSM) was used to survey the response of culture conditions (temperature, degrees brix, time, and initial iron concentration) to the bioaccumulation of ferric ion (Fe^{3+}) in yeast (*Saccharomyces pastorianus*). On the other hand, the Box-Behnken design was used to determine the optimum conditions of 24°C, 13°Bx of the culture solid content for 49 h incubation and 656 ppm of initial iron concentration. The total Fe^{3+} content in the biomass was significantly affected by the culture temperature and degrees brix ($p<0.0001$). Under optimum conditions, the maximum level of Fe (III) ions in the dry cell weight of *S. pastorianus* was 16.82±0.65 mg/g. The results from statistical analysis showed that the model was significant ($p<0.0001$) and adequate.

Keywords: **Box-Behnken, Fe (III) citrate, optimization, response surface methodology, *Saccharomyces pastorianus*.**

Classification numbers: **2.2, 3.5**

Introduction

Iron is known to be an essential micronutrient for all living organisms as it takes part in biochemical functions such as oxygen transportation, molecules storage, and enzyme catalysation, which require the redox reaction for the generation of energy, metabolism, and immune systems [1]. Iron deficiency (ID) and iron deficiency anaemia (IDA) are popularly considered as nutritional problems.

The most extensive cure for ID and IDA is oral iron medication. The most prescribed iron supplement contains ferrous salts that have quite low bioavailability rates [2]. Therefore, it is necessary to investigate the bioavailability and safest form of iron and identify the optimal length of treatment to supply enough iron for the body's needs.

An interest in the methods of the production of microelements such as enriched yeast has increased dramatically in recent years to produce products to prevent trace element deficiencies that are non-toxic, have high availability, are easy to digest, and are easily absorbed by the human body. The ability to bio-absorb metal elements turns yeast into a host for many mineral supplements. Recently, *Saccharomyces cerevisiae* yeast cells have been used to survey metal transportation and accumulation. Under proper conditions, *S. cerevisiae* can absorb a considerable quantity of trace elements including iron, zinc, copper, manganese, and selenium, which can be synthesized into organic compounds [3-5].

Saccharomyces pastorianus is a key component to the fermentation of lager beer and grows facultatively in popular media. As a successful combination of a variety of Saccharomyces strains, *S. pastorianus's* ability of bio-absorption and conversion of iron in media are as high as that of *S. cerevisae*. Ferric citrate pentahydrate

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($C_6H_5FeO_7.5H_2O$) is chosen to regulate the blood levels of iron because of insignificant harmful effects of gastric acidity to the yeast growth [6].

Response surface methodology (RSM) is a collection of mathematical and statistical techniques that is useful for designing experiments, establishing models, and analysing the effects of several independent factors [6]. RSM assists in the limitation of the number of experimental trials needed to evaluate multiple factors and their interactions. RSM is based on the fit of empirical models to experimental data obtained in relation to the experimental design, which leads to the employment of polynomial functions to express the system as an equation and to display experimental conditions until its optimization [7]. Researchers can use RSM to study responses related to several variables. The results of the interaction between levels of variables are the key to adjusting the factors to achieve the optimum condition. The result of analysis of the RSM's statistical data can predict the values of a given response with any combination of factors. Response surface methodology is chosen to aid this study based on the advantages of narrowing down the number of experimental runs and surveying the connection between responses and variables. The Box-Behnken technique allows the user to set the number of levels as well as factors related to experiment to create an ideal model. The Box-Behnken design (BBD) is proven to be the most popular technique to optimize processes thanks to the availability of the theory and fundamentals of BBD [6, 8]. BBD is designed for four-variable optimization with 27 experimental runs (3 central points) [7]. To compare with other designs only using one factor, it is noted that BBD can assist researchers in the study of an extensive number of variables over a small number of experimental runs because of its efficiency and economy, thereby reducing the quantity of experimental runs, reagents, samples, and effort to determine the optimal conditions for bio-absorption. Further, BBD improves the statistical interpretation and demonstration of the interaction between variables.

The present study focuses on optimising the amount of *Saccharomyces pastorianus* biomass cultivated in control and experimental media enriched with iron under culture operating parameters (e.g. temperature, culture solid content (degrees brix), incubation time, and initial iron concentration) for producing a yeast biomass with high iron content. Then, RSM is employed with a four-variable, three-level BBD to investigate the affinities between the absorption variables and the content of Fe (III) ions in dry cell weight (mg Fe per g dry cell weight). Through that process, the optimal cultivation parameters for the highest content of Fe (III) ions in the biomass were determined. The optimum absorption conditions can be used for the development of iron-enriched *S. pastorianus* for human and animal health on both laboratory and industrial scales in pharmaceutical and functional food production.

Materials and methods

Microorganism and media

S. pastorianus was obtained from the Research Institute of Brewing and Malting (Czech Republic) under freeze drying condition. It was then reactivated and cultured in a sterilized medium to obtain 10^8 CFU/ml. The pale malt obtained from the Barrett Burston Malting Co. Pty. Ltd. (Australia) contained 10.2% of protein and 178 mg/l of beta-glucan. Based on the beer production process of Beer & Malt Manufacturing, the 20°Bx malt wort was produced following the procedure of Esslinger [3]. The degrees of brix were adjusted in various levels depending on the experimental plan.

Chemicals and apparatus

Iron (III) citrate pentahydrate ($C_6H_5FeO_7.5H_2O$) and other chemicals used for analytical grade were purchased from Merck (Millipore, USA). Hydroxylamine hydrochloride ($NH_2OH.HCl$), sodium acetate (CH_3COONa), and 1,10 - phenanthroline was also obtained from Sigma-Aldrich Co. (USA) as a calibration standard for UV-Vis analysis. To obtain a stock Fe (III) citrate solution of 10000 ppm Fe, 60 g of $C_6H_5FeO_7.5H_2O$ was dissolved in 1 litre of deionized water at pH 4.0. The stock solution was further diluted to obtain the desired initial concentrations, from 500 to 1000 mg/l, which were sterilized (120°C, 10 min).

Fermentation experiments

Culturing experiments were run in triplicate in 250 ml Erlenmeyer flasks by using the malt media to optimize cultivation conditions. The 250 ml Erlenmeyer flasks containing 200 ml of wort medium were inoculated with 10% (v/v) of a previously prepared yeast sample. Fe (III) citrate pentahydrate salt ($C_6H_5FeO_7.5H_2O$) was added to the medium at the initial concentrations of 500-1000 ppm in the preliminary studies. The cultures were aerobically incubated on a rotary shaker at 400 rpm. The samples were withdrawn at regular intervals and analysed to determine the dry cell weight (g/l) and Fe (III) ions uptake (mg/g).

Determination of iron absorption

The incorporation of Fe^{3+} into the yeast cells was determined by two steps using the chemical method of cell lysis. Firstly, to remove the free Fe^{3+} ions bound to the cell surface and iron precipitation, the biomass was washed with deionized water and then filtrated by vacuum filter. Secondly, 1 g of the filtrate was digested using HNO_3 65% for 30 min and heated to 150°C to release the intercellular iron. The iron content in the yeast cells was analysed using a UV-Vis spectrophotometer. The standard curve was prepared with samples obtained by the dilution of the standard solutions (1000 ppm) with deionized water.

Dry cell weight measurement

For measurement of the dry cell weight, 200 ml of culture after planned fermentation was centrifuged at 3000 rpm for 5 min and washed twice with deionized water. The biomass was freeze dried and weighed to determine the dry cell weight.

Experimental design

The Box-Behnken design provides the quantity of experimental runs following this equation:

$$N = 2k^2 - 2k + cp \quad (1)$$

where k is the factor number and cp is the replicate number of the central point. In RSM, the collected data from variables is of practical use for solving equations and for concluding the optimum condition. To be more specific, an empirical second order polynomial model is fitted to the data for analysis of the variables as shown in Eq. 2:

$$Y = \beta_0 + \sum_{i=1}^{k} B_i X_1 + \sum_{i=1}^{k} B_{ii} X^2 + \sum_{i=1}^{k} \sum_{j=i+1}^{k} B_{ij} X_i X_j \quad (2)$$

where β_o is a constant, B_i the linear coefficient, B_{ii} the quadratic coefficient, and B_{ij} is the cross-product coefficient. The variables X_i and X_j are levels of the independent variables while k equals the number of the tested factors (k=4). The optimal culture conditions for the Fe (III) ions in dry cell weight (Y) were determined using RSM to study the effects of culture parameters on the accumulation yields of the final biomass and intercellular iron content in yeast cells.

A summary of the temperature, degrees brix, incubation duration, and initial Fe (III) ions concentration of the 27 experiments is shown in Table 1.

Table 1. Experimental independent variables.

Factor	Units	Levels and range (coded)		
		-1	0	+1
Temperature	°C	20	26	32
Degrees brix	°Bx	12	14	16
Incubation time	hour	24	48	72
Initial Fe (III) ions concentration	ppm	500	750	1000

Model fitting and statistical analysis

The experimental data was analysed by using Design Expert software version 11 (Stat-Ease, Inc., Minneapolis MN, USA) for regression analysis and statistical significance of the derived equation. A design of 27 experiments was formulated from four factors (2^4), three replicates at the central points, and the three levels are used in experiment based on the order of Eq. 2. The results of the equation and of the response surface plots help to optimize reasonable values. The adequacy of the fitted model was counted using the lack of fit, the coefficient of determination (R^2), and the statistical significances of all terms in the polynomial model by an F-test from ANOVA at a probability (p) of 0.001, 0.01, or 0.05, then calculating the determined coefficients to achieve curve maps from the regression models.

Results and discussion

The results obtained from experiment (Table 2) showed the effects of temperature, incubated time, degrees brix, and initial iron (III) concentration on the accumulation of Fe in yeast biomass. The second-order polynomial equation presented the connection between the variables and responses and the optimum conditions of the process were obtained from the model by fitting the equation with coefficients. Table 3 showed the results of the ANOVA with regression model. A probability value smaller than 0.05 indicated that the model terms are significant. An insignificant value of "lack of fit" showed the validity of the quadratic model for accumulation by *S. pastorianus*. Equation 3 lists the final empirical formula model for the amount of Fe (III) ions in dry cells weight in terms of coded factors:

$$\text{Iron (III) ions in dry cells weight (mg/g)} = +15.68 - 1.80A - 2.71B + 0.33C - 0.589D + 1.76AB - 0.703AC - 0.810AD - 0.490BC + 0.775BD + 1.95CD - 2.51A^2 - 3.63B^2 - 4.44C^2 - 0.879D^2 \quad (3)$$

where A, B, C and D are the coded terms for temperature, degrees brix, time and initial Fe (III) concentration.

Table 2. Comparison of experimental and predicted values on Fe (III) ions in dry cell weight (mg/g).

Std order	Independent variables				Fe (III) ions in dry cell weight (mg/g)*	
	Temp (°C)	Degrees brix (°Bx)	Time (h)	Initial iron (III) centration (ppm)	Experimental value	Predicted value
1	20	12	48	750	15.89	15.82
2	32	12	48	750	9.05	8.69
3	20	16	48	750	6.78	6.88
4	32	16	48	750	6.98	6.80
5	26	14	24	500	12.21	12.58
6	26	14	72	500	9.51	9.33
7	26	14	24	1000	7.57	7.49
8	26	14	72	1000	12.68	12.06
9	20	14	48	500	13.51	13.88
10	32	14	48	500	12.15	11.89
11	20	14	48	1000	14.02	14.32
12	32	14	48	1000	9.42	9.10
13	26	12	24	750	9.23	9.50
14	26	16	24	750	5.52	5.07
15	26	12	72	750	10.65	11.14
16	26	16	72	750	4.98	4.75
17	20	14	24	750	10.01	9.50
18	32	14	24	750	6.91	7.31
19	20	14	72	750	11.75	11.57
20	32	14	72	750	5.84	6.56
21	26	12	48	500	15.67	15.25
22	26	16	48	500	8.15	8.28
23	26	12	48	1000	12.43	12.52
24	26	16	48	1000	8.01	8.65
Repeated runs						
25	26	14	48	750	16.04	15.68
26	26	14	48	750	16.02	15.68
27	26	14	48	750	14.98	15.68

*Average value of triplicate experiments.

Table 3. Analysis of variance (ANOVA) for Fe (III) ions in dry cell weight (mg/g).

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value	Comment
Model	312.44	14	22.32	66.52	<0.0001	
A-Temperature	38.92	1	38.92	116.00	<0.0001	
B-Degrees brix	88.02	1	88.02	262.37	<0.0001	
C-Incubation time	1.31	1	1.31	3.90	0.0719	
D-Initial Fe concentration	4.17	1	4.17	12.42	0.0042	
AB	12.39	1	12.39	36.93	<0.0001	
AC	1.97	1	1.97	5.88	0.0320	
AD	2.62	1	2.62	7.82	0.0161	
BC	0.9604	1	0.9604	2.86	0.1164	significant
BD	2.40	1	2.40	7.16	0.0202	
CD	15.25	1	15.25	45.45	<0.0001	
A^2	33.50	1	33.50	99.86	<0.0001	
B^2	70.18	1	70.18	209.19	<0.0001	
C^2	105.02	1	105.02	313.05	<0.0001	
D^2	4.12	1	4.12	12.28	0.0044	
Residual	4.03	12	0.3355			
Lack of fit	3.29	10	0.3291	0.8952	0.6352	not significant
Pure error	0.7352	2	0.3676			
Cor total	316.46	26				
R^2=0.9873, R^2 adj=0.9724; C.V%=5.47; Adeq precision=25.639						

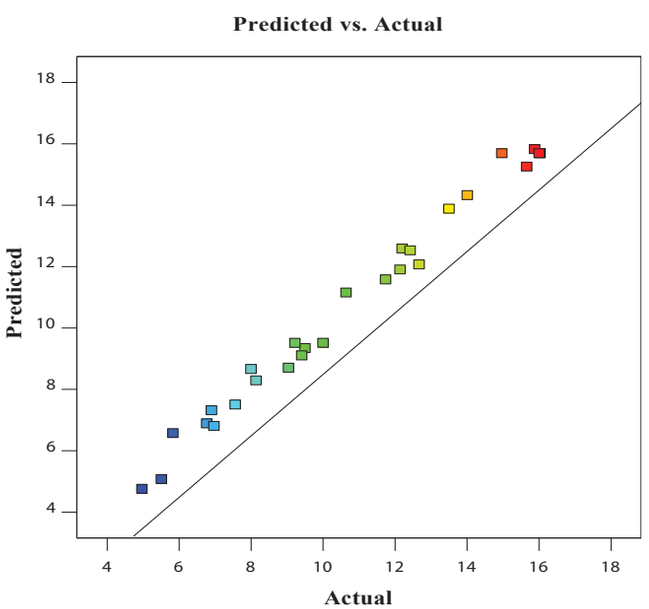

Fig. 1. Plot of experimental versus predicted values of Fe (III) ions amounts per dry cell weight of *S. pastorianus*.

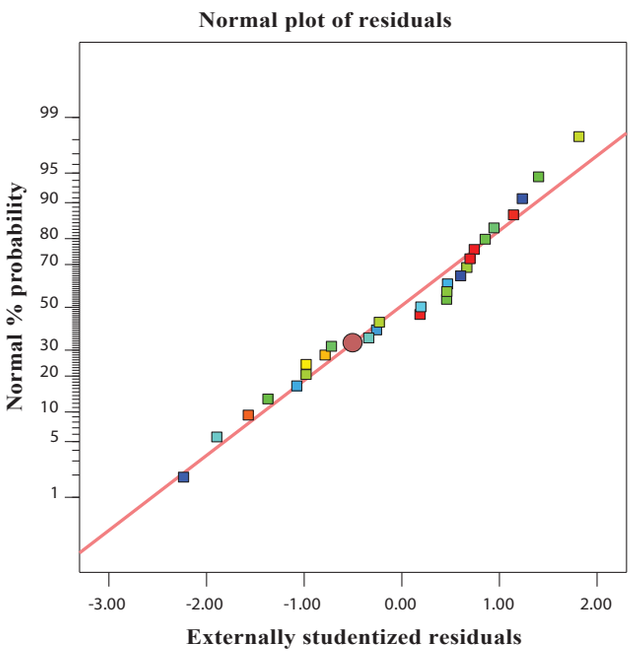

Fig. 2. Plot of experimental residual versus predicted values of Fe (III) ions amounts per dry cell weight of S. *pastorianus.*

The results from ANOVA (Table 3) also identified that the second-order polynomial model of Eq. 3 for accumulation yields of Fe (III) ions was statistically significant and adequate to represent the actual relationship between responses and the variables, with a small probability value ($p<0.0001$) and satisfactory coefficient of determination ($R^2=0.9873$). The R-squared (R^2) and adjusted R-squared (R^2adj) coefficients of this model are 0.9873 and 0.9724, respectively, which are close to 1.0. This indicates a good fit of the model with the experimental data. Moreover, the resemblance between the R^2 and adjusted R^2 reveals the ability of the model to anticipate the results of the optimization. The F-values of 66.52, together with $p<0.0001$ for the Fe (III) ions in dry cells weight, and insignificant p-value for "lack of fit" as 0.6352 for the accumulation of Fe (III) ions yield, indicated that the model adequately fit the experimental data. Furthermore, the normal probability plot and predicted versus actual plot are presented in Figs. 1 and 2. The examination of the presumption of homogeneity is conducted by using the residuals. In particular, the studentized residuals are plotted against the probability values. The model's suitability level for the present research is indicated by the data in regard to either side of the zero line, which is spread out in a homogeneous manner.

Additionally, the predicted versus actual plots were delineated between predicted and actual response parameter values. These diagnostic plots were made in order to investigate the goodness of fit of the proposed model. The predicted R^2 (0.9349) shows an appropriate harmony between the value predicted by the model and the actual data. Moreover, the absence of trends in the plot of studentized residual versus the values predicted by the model shows that the variances in the data are acceptable and no outliers are present in the experiments (Fig. 2). Fig. 1 shows that the predicted versus actual plot revealed a highly linear trend through the origin, which signified that the experimentally observed values of Fe(III) ions in dry cell weight were in close agreement with predicted values. From Table 3, the overall effects of the four manipulated variables on the response revealed that the temperature and degrees brix ($p<0.0001$) were important factors for the accumulation of Fe (III) ions in dry *S. pastorianus* cells.

Response surface analysis

Using surface response plots of the polynomial model, the relationships between the culture conditions and the response could be better understood by holding two variables constant at its central level and studying the relationship between the other two variables in the experimental range under investigation. To express the effects of any independent variable on the absorption of Fe (III) ions, three-dimensional surface plots were generated according to Eq. 3. On the basis of a quadratic polynomial of the response surface methodology, the effect of interacting variables, i.e., temperature (14-32^{o}C), incubated time (24-72 h), degrees brix (12-16^{o}Bx) and initial iron (III) concentration (500-1000 ppm) on the accumulation of Fe (III) were analysed.

The information obtained from the experiments showed that the range of ferric ions in dry weight was 5.52 to 16.04 (mg/g). The lack of fit F-value of 0.8952 suggested that the lack of fit is not significant, which presents acceptability of the model. Furthermore, the relative standard deviation (RSD) or C.V.% of 5.47 indicates the accuracy and repeatability of the model. Eventually, in this study, the modified quadratic polynomial equation is an appropriate model to explain the level of ferric ion amounts entrapped in *S. pastorianus* cells. The regression analysis report for the modified quadratic model showed that linear, squared, and interaction coefficients are significant ($p<0.05$) except for one linear interaction (Table 3). It is reported that the

coefficients of the three factors (temperature, degrees brix, and initial iron concentration) are negative, whereas positive coefficients were obtained for the factor of incubated time. The negative coefficient of the three factors on response implies that lowering these factors causes higher iron content in dry cell weight, which affects the number of cellular active sites available to uptake as well as metal speciation.

In Fig. 3, the relationship between the initial iron concentration and three remaining parameters was investigated. This plot shows that the interaction between initial iron concentration and temperature, degrees brix, and time is insignificant. The effect of initial iron concentration as a significant factor is relatively lower than that of the other variables. Previous studies [9] supposed that fermentation temperature had a close relationship with the growth of yeast and Fe (III) ions was a biomass-related product. The temperature disturbs the physical or chemical structure of the channels, which could either enhance or decline the amount of metal bioaccumulation sites. Besides, the malt solution obtained not only contains mainly carbohydrates (glucose, fructose, sucrose, maltose, maltotriose, and non-fermentable dextrins) but also many other molecular compounds such as nitrogenous materials and other constituents too numerous to mention [10]. Consequently, the higher the wort gravity, the smaller the impact on the bioaccumulation and biomass production because a great amount of large molecular compounds present in the malt wort is capable of binding ions, which lowers their bioavailability [3, 11]. The metabolism of Saccharomyces strains relies on glucose as the key source of energy and through fermentation the cells uptake glucose along with trace elements, which is iron in this work, to produce ethanol or pyruvate. Furthermore, the growth behavior of *S. pastorianus* has not been shown to be significantly different from other strains of Saccharomyces while its metabolism requires a fixed amount of iron for the citric acid cycle and respiratory chain. The iron concentration suitable for *S. cerevisiae* is reported to be between 5 ppm to 1500 ppm. In this work, we choose 1000 ppm Fe supplemented in the form of $C_6H_5FeO_7.5H_2O$ to study for *S. pastorianus*. The results of this work point out that supplementation of malt media with Fe^{3+} has a significant influence on the bioaccumulation rate and brewing yeast growth. Iron at higher, semi-toxic levels (above 750 ppm) act as a stressing agent and cause growth inhibition as the decrease of Fe

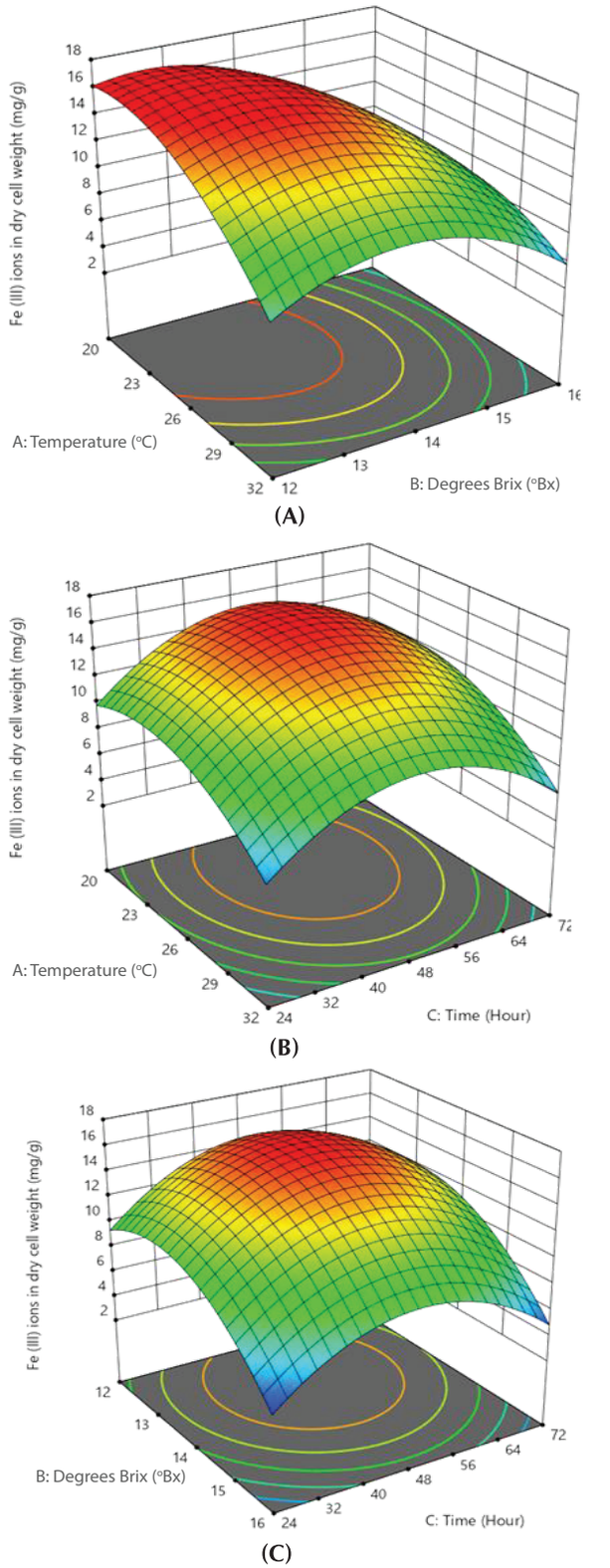

Fig. 3. Response surface plot indicating the effect of (A) temperature (°C) and degrees brix (°Bx), (B) temperature (°C) and incubated time (hour), (C) degrees brix (°Bx) and incubated time (h) interaction on Fe (III) ions amount per dry cell weight of *S. pastorianus*.

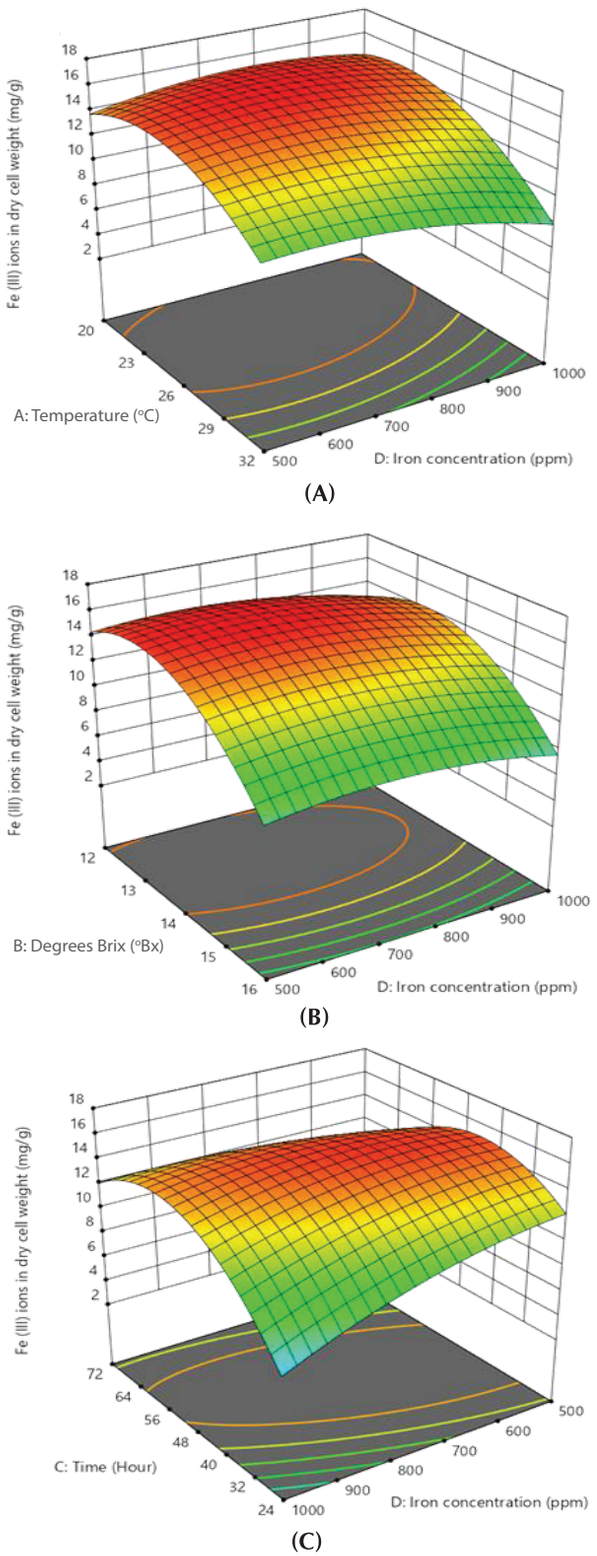

Fig. 4. Response surface plot indicating the effect of (A) temperature (°C) and initial iron concentration (ppm), (B) degrees brix (°Bx) and initial iron concentration (ppm), (C) incubated time (hour) and initial iron concentration (ppm) interaction on Fe (III) ions amount per dry cell weight of *S. pastorianus*.

(III) ion accumulation elongates the fermentation time. The ability to adjust the amount of iron intake helps yeast to control nutrient concentration, including metal levels, in its cells. Increasing the concentration of $C_6H_5FeO_7.5H_2O$ in the medium up to 1000 ppm (500, 750, 1000 ppm) resulted in a rising trend of iron accumulation in the biomass of the yeast *S. pastorianus*. The highest quantity, about 16 mg Fe g^{-1} dry wt., was obtained at 750 ppm Fe. *S. pastorianus* is proven in this study to be a promising replacement of *S. cerevisiae* based on the advantages of its fermentation process, which could be more appropriate for use in animal feed. Since *S. pastorianus* is a yeast in the GRAS group, its use in functional food for human is another aspect worth further study [12].

One of the most efficient tools in the analysis of interactions is using graphical plots, especially three-dimensional response surface plots. In Fig. 4, the relationship between the temperature, degrees brix, and time investigated while the initial iron concentration was constant at the median value. This plot indicates that the quadratic coefficients of temperature, degrees brix, and time are significant. As a result, the lower the degree brix, incubation time, and temperature, the higher the impact on the bioaccumulation and biomass production. By raising degree brix, incubation time, and temperature, the enriched cells of *S. cerevisiae* increased to an optimum point and then decreased afterward.

Determination of optimum conditions

The optimal conditions used for the accumulation test for Fe (III) citrate were determined using the maximum desirability to verify the predictive capacity of the model. Results of the optimal conditions to obtain the highest accumulation yield of Fe (III) ions from Fe (III) citrate were a temperature of 24°C, degrees brix of 13°Bx, time of 49 h, and initial iron concentration of 656 ppm. After the performance of triplicate experiments under optimum conditions, the experimental value was 16.82±0.65 mg Fe/g dry cells weight for Fe (III) ion absorption. These experimental results are in good agreement with the predicted value for Fe (III) ion accumulation (16.91±0.58 mg Fe/g dry cells weight).

Conclusions

In this study, the optimum condition leads to high iron absorption efficiency into *S. pastorianus,* which is

demonstrated by using BBD. The bioaccumulation of ferric ions is influenced by all four factors in which the incubation temperature and degrees brix have significant effects on the iron absorption ability of the yeast as well as the biomass formation. A decrease of iron absorption at high degree brix and high temperature was observed in this study. An optimum condition for bioaccumulation of ferric ions of 16.8 mg/g biomass was achieved with BBD using RSM at 656 ppm initial iron concentration in 13°Bx medium and with *S. pastorianus* incubated at 24°C for 49 h. Furthermore, the result verified that the ability of iron absorption of *S. pastorianus* is limited based on the limited number of active sites for absorbing ferric ions inside the cells, so it is not effective to supply a larger amount of initial iron concentration to the growth medium. It is concluded that iron-enriched *S. pastorianus* has the potential for mass production with a low-cost natural source with the recommended optimum conditions found in this study.

The authors declare that there is no conflict of interest regarding the publication of this article.

REFERENCES

[1] W. Bahafid, et al. (2017), "Yeast biomass: an alternative for bioremediation of heavy metals", *Yeast-Industrial Applications*, DOI: 10.5772/intechopen.70559.

[2] F. Gaensly, et al. (2014), "The uptake of different iron salts by the yeast Saccharomyces cerevisiae", *Brazilian Journal of Microbiology*, **45(2)**, pp.491-494.

[3] A. Poreda, and T. Tuszyński (2007), "Influence of magnesium and zincions on trehalose synthesis and fermentation activity in brewing yeast Saccharomyces carlsbergensis", *Chemia i Inżynieria Ekologiczna*, **14(2)**, pp.197-207.

[4] I. Schröder, E. Johnson, S. De Vries (2003) "Microbial ferric iron reductases", *FEMS Microbiology Reviews*, **27(2-3)**, pp.427-447.

[5] Y. Yuan, et al. (2004), "Construction of a high-biomass, iron-enriched yeast strain and study on distribution of iron in the cells of Saccharomycescerevisiae", *Biotechnology Letters*, **26(4)**, pp.311-315.

[6] G. Fan, et al. (2008), "Optimizing conditions for anthocyanins extraction from purple sweet potato using response surface methodology (RSM)", *LWT-Food Science and Technology*, **41(1)**, pp.155-160.

[7] Y. Azila, M. Mashitah, S. Bhatia (2008), "Process optimization studies of lead (Pb (II)) biosorption onto immobilized cells of Pycnoporus sanguineus using response surface methodology", *Bioresource Technology*, **99(18)**, pp.8549-8552.

[8] B. Szulc-Musioł, B. Dolińka, F. Ryszka (2016), "Influence of incubation conditions on baker's yeast enrichment in selenium", *Journal of Elementology*, **21(4)**, pp.1253-1261.

[9] R. Singh, et al. (2010), "Biosorption optimization of lead (II), cadmium (II) and copper (II) using response surface methodology and applicability in isotherms and thermodynamics modeling", *Journal of Hazardous Materials*, **174(1-3)**, pp.623-634.

[10] Y. He, et al. (2014), "Wort composition and its impact on the flavour-active higher alcohol and ester formation of beer-a review", *Journal of the Institute of Brewing*, **120(3)**, pp.157-163.

[11] T. Satyanarayana, G. Kunze (2009), *Yeast Biotechnology: Diversity and Applications*, Springer, pp.744.

[12] M. Amiri, et al. (2017), "Optimization of culture conditions for enrichment of saccharomyces cerevisiae with Dl-α-Tocopherol by response surface methodology", *Iranian Journal of Pharmaceutical Research (IJPR)*, **16(4)**, pp.1546-1554.