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MATHEMATICAL MODELING OF THE NUTRIENT MEDIUM COMPOSITION FOR THE PRODUCTION OF YEAST PHYTASE

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Abstract

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The objective of the present study was the optimization of the nutrient medium composition for *Candida melibiosica 2491* cultivation by means of mathematical methods of modeling and production of yeasts with higher enzyme capacity. An optimal composition plan was used for the mathematical description of the process that enabled the generation of linear and non-linear regression models with a minimum number of trial observations. The mathematical modeling resulted in a new composition of the nutrient medium for the phytase biosynthesis of *C. melibiosica*, namely: (g/dm^3) fructose – 30.0; yeast extract – 5.0; meat peptone – 8.71 and $\hat{E}\acute{I}_2\eth\hat{I}_4$ – 0.2 mmol, achieving an increase of cell phytase productivity with 22.5%.

Key words: mathematical modeling, yeasts, phytase, nutrient medium

Introduction

The phytase enzyme has been thoroughly studied in the resent years because its use leads to the reduction of phytate contents in plant animal feed and human food. The phytic acid [*myo*-inositol (1, 2, 3, 4, 5, 6) hexakisphosphate], respectively phytates, are found mainly in cereal, leguminous and oil-bearing seeds and grains, hence, in the majority of foods of plant origin. The phytate content (mg/g DW) varies within 9.8 – 21.3 in maize; 12.7 – 21.6 in wild rice; 5.9 – 11.8 in sorghum; 3.2 – 7.3 in whole wheat bread; 1.9 – 4.3 in whole rye bread; 4.3 – 8.2 in maize bread and 39.3 – 57.2 in toasted sesame seeds (Greiner and Konietzny, 2006).

A large portion – 50% to 80% of the total organic phosphorus (Pi) content is found in bonded form in *myo*-inositol phosphate and is non-assimilable by monogastric animals such as swine, poultry, fish and humans, etc., because the phytase activity of their food digestion tract is either missing or at very low levels (Lott et al., 2000). That is why the diets of these animals are supplemented with inorganic Pi mostly as $Ca_3(PO_4)_2$. The phytate Pi that is not assimilated by these animals causes environmental issues as it accumulates in manure and water, gets hydrolyzed by soil

and water microorganisms and causes eutrophication and, ultimately, destruction of the flora and fauna. Moreover, Ca, Mg, Zn, Fe and other elements are chelated in the phytates that makes them non-assimilable (Žy³a, 1994; Liu et al., 1998; Leu and Stahl, 2001).

The phytase [*myo*-inositol (1, 2, 3, 4, 5, 6) hexakisphosphate phosphohydrolase] catalyzes the phytate hydrolysis step by step to *myo*-inositol phosphates with a different rate of phosphorylation and H_3PO_4. Depending on the location of carbon in the *myo*-inositol ring, where dephosphorylation is initiated, they are divided into: 3-phytases (E.C. 3.1.3.8), 5-phytases (E.C. 3.1.3.72) and 6-phytases (E.C. 3.1.3.26) (Greiner and Konietzny, 2006). Phytase is found in low levels in plants and some animal tissues, therefore, microorganisms are of considerable importance for its study and production.

Scientific literature provides information on phytase yeast producers of the genera *Arxula* (Sano et al., 1999), *Pichia* (Nakamura et al., 2000), *Saccharomyces* (Zyla, 1994), *Candida* (Georgiev and Gargova, 2006) and *Schwanniomyces* (Lambrechts et al., 1992). The supplementation of yeast cell-bonded phytase to food and animal feed increases the assimilation rate of phosphorus and minerals. On the other hand, the production of different *myo*-inositol phosphates, the functions of which are not fully studied from medical point of view, is of scientific and practical importance.

The mathematical methods of experimental planning use a relatively small number of observations, resulting in an analytical expression accounting for the effect on the studied process of each separate factor, their interrelations as well as those at a higher level. Thus, Sariyska et al. (2002) achieved a 40% increase of enzyme productivity by means of a linear model of the nutrient medium composition and Bogar et al. (2003) and Blazheva et al. (2005) optimized the conditions of enzyme biosynthesis by a central (respectively, optimal) composition plan and achieved an increase of the microbial process enzyme activity with 50%, respectively, 13%.

The objective of the present study was to optimize the composition of the nutrient medium for the cultivation of *Candida melibiosica* 2491 by means of mathematical modeling methods for the production of yeasts with increased enzyme activity.

Material and Methods

Microorganism

A screening procedure was used for the selection of the strain *Candida melibiosica* 2491 that is perspective for intracellular phytase biosynthesis. It was maintained at a temperature of 4°Ñ on nutrient medium agar slants, a modified medium of Sano et al, (1999) in which glucose was replaced by fructose (Georgiev and Gargova, 2007).

Nutrient medium and cultivation conditions

For the submerged cultivation of the strain was used in a nutrient medium with the following primary composition ($g.dm^{-3}$): fructose – 20, yeast extract – 10, meat peptone – 10 and KH_2PO_4 – 0.2 mmol (0.62 mg% Pi) and initial ðÍ value of 5.5. The concentration of carbon and phosphorus sources in this medium were identified after a series of monofactorial experiments, in which an activity of 8.6 U./g DW^{-1} (absolute dry biomass) was achieved (Georgiev and Gargova, 2007). Medium culturing was done with yeast suspension of a 24–h culture with an optical density $OD_{600} = 0.68$. The submerged cultures took place in 300 cm^3 Erlenmeyer flasks, containing 30 cm^3 of the nutrient medium at 28°C for a period of 30 h on a rotary shaker with 220 min^{-1}.

Phytase activity (PhA)

After the end of the culturing process, the biomass was separated by centrifuging at 10 000 min^{-1}, the sediment was washed twice with 0.2 M sodium acetate buffer solution with pH 5.5 and resuspended in 5 cm^3 of the same buffer. The enzyme activity was identified by the method of Zyla (1994) that uses whole cells and the inorganic phosphate – by the method of Engelen et al. (1994). One unit of phytase activity was defined as the quantity of enzyme that catalyzed the hydrolysis of 0.5 mmol of sodium phytate

solution (Sigma, P-3168) with the formation of 1 μmol inorganic orthophosphate at 45°C and pH 4.5 and was expressed in E.(g ADB)$^{-1}$. The extinction of the colored solution, containing ammonium heptamolybdate (Scharlau, AM0349) and ammonium vanadate (Scharlau, AM0467) was measured at wave length of 415 nm with Spekol 11.

Experiment planning

An optimal composite plan was used for the mathematical description of the process (Mason et al., 2003) that enabled the generation of linear and non-linear regression models with a minimum number of experimental observations:

$$Y_{\text{mod}} = b_0 + \sum_{i=1}^{k} b_i . x_i + \sum_{i=1}^{k} b_{ii} . x_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} b_{ij} . x_i . x_j, \quad (1)$$

Coefficients b_i, b_{ij} and b_{ii} recorded the effect of, respectively, each factor - x_i; their interrelationships - $x_i . x_j$ and those squared - x_i^2 and k was the number of factors.

The statistical processing of the experimental data and the analysis of the results were done in ANOVA (Microsoft Excel 2003). The computation and optimization procedures were effected on Eureka (2000) and Matlab (Mathews and Fink, 2001) software. Some calculations were done with our own algorithms. The graphic presentation was done on Microsoft Excel 2003 and Sigma plot 9.0.

The variation intervals of the nutrient medium components were identified after a series of monofactorial experiments. The real and coded values of the independent variables are presented in Table 1. The primary data base for Y_{exp} was formed after averaging the results of three independent observations for each combination of factors.

Results and Discussion

The matrix of the experiments for an optimal composition plan on three variation levels of four factors, (± 1) the experimental results on $PhA - Y_{exp}$ and those from equation (2) – Y_{mod} are presented in Table2. The analytical expression of the obtained regression equation is as follows:

$$Y_{mod} = 4.60+1.08.X_1-0.812.X_2-0.573.X_3-0.746.X_1.X_2+0.595.X_2.X_3+0.573.X_1.X_2.X_3+1.61.X_1^2+1.13.X_2^2-1.35.X_3^2-0.584.X_4^2 \quad (2)$$

The mathematical model is adequate at a confidence level α=0.05 and freedom degree ν=10 (Table 3).

The analysis of the regression equation lead to the following conclusions:

- the independent influence of the carbon source X_1 vs. that of b_o in the target function was 23.5%, while the influence of the same factor on phytase activity was expressed stronger with its participation as a coefficient in front of X_1^2 - 35%. Since both coefficients were positive, we assumed that even a small increase of the concentration of the carbon source would have a favorable effect on the increase of the enzyme activity according to the linear and non-linear law;

- the participation of X_2 in Y_{mod} was 18% but with a minus sign. At a first approximation we could assume that $|b_2| \cong b_1$. However, the coefficient in front of X_2^2 was positive and its weight in the response function was 24.6%. Obviously, even the lowest concentration of the yeast extract would satisfy the needs of *Candida melibiosica* 2491 in bioactivators of the enzyme synthesis. The high values of X_2 (within 0 and +1) probably lead to the suppression of strain productivity;

- X_3 and X_3^2 participated in (2) – 12.5 and 29.4%, respectively. Considering their signs, the conclusion

Table 1

Factor	Coded value		
	-1	0	1
X_1-fructose, g.dm^{-3}	10	20	30
X_2-yeast extract, g.dm^{-}	5	10	15
X_3-meat peptone,	8	10	12
X_4-KH_2PO_4, mmol	0.1	0.2	0.3

Table 2

№	X_1	X_2	X_3	X_4	X_1X_2	X_1X_3	X_1X_4	X_2X_3	X_2X_4	X_3X_4	$X_{12}X_3$	$X_{12}X_4$	$X_{13}X_4$	$X_{23}X_4$	X_1^2	X_2^2	X_3^2	X_4^2	Y_{exp}, g (g ADB)$^{-1}$	Y_{mod}, g (g ADB)$^{-1}$
1	-1	-1	-1	-1	1	1	1	1	1	1	-1	-1	-1	-1	1	1	1	1	5.12	4.99
2	-1	-1	-1	1	1	1	-1	1	-1	-1	-1	1	1	1	1	1	1	1	4.91	4.99
3	-1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	1	1	1	1	1	3.75	3.8
4	-1	-1	1	1	1	-1	-1	-1	-1	1	1	1	-1	-1	1	1	1	1	4.69	3.8
5	-1	1	-1	-1	-1	1	1	-1	-1	1	1	1	-1	1	1	1	1	1	6.25	4.81
6	-1	1	-1	1	-1	1	-1	-1	1	-1	1	-1	1	-1	1	1	1	1	3.5	4.81
7	-1	1	1	-1	-1	-1	1	1	-1	-1	-1	1	1	-1	1	1	1	1	4.17	3.71
8	-1	1	1	1	-1	-1	-1	1	1	1	-1	-1	-1	1	1	1	1	1	4.17	3.71
9	1	-1	-1	-1	-1	-1	-1	1	1	1	1	1	1	-1	1	1	1	1	9.78	9.78
10	1	-1	-1	1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1	1	1	9.67	9.78
11	1	-1	1	-1	-1	1	-1	-1	1	-1	-1	1	-1	1	1	1	1	1	6.22	6.3
12	1	-1	1	1	-1	1	1	-1	-1	1	-1	-1	1	-1	1	1	1	1	5.33	6.3
13	1	1	-1	-1	1	-1	-1	-1	-1	1	-1	-1	1	1	1	1	1	1	4.46	4.33
14	1	1	-1	1	1	-1	1	-1	1	-1	-1	1	-1	-1	1	1	1	1	4.16	4.33
15	1	1	1	-1	1	1	-1	1	-1	-1	1	-1	-1	-1	1	1	1	1	5.14	5.52
16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	4.92	5.52
17	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	9.44	7.28
18	-1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	3.19	5.13
19	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	4.88	4.92
20	0	-1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	6.8	6.54
21	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2.93	2.68

Table 3

Parameter	df	SS	MS	F	Significance F
Regression	10	72.45077	7.245077	6.074994	0.001308675
Residual	14	16.69649	1.192607		
Total	24	89.14726			

would be that the maximum value of Y_{mod} for this factor would be within $\pm$ 1 (Òable 1);

- the coefficient in front of $X_1.X_2$ was negative thus supporting the assumption on the variation range of X_2. It also defined the variation range of X_3 – about its minimum value, since $b_{23}>0$;

- the participation of X_1, X_2 and X_3 in (2) as linear and non-linear members with the respective sign was relatively equal, which showed their definitive role in the biosynthesis of the target product;

- X_4 participated in the regression equation only as a squared member. However, it was essentially important for *Candida melibiosica* 2491 development and productivity. From a mathematical point of

view, the response function would achieve its maximum at X_4=0, which coincided with the data from the monofactorial experiment.

Those contemplations confirmed the significance of the obtained results and showed that the choice of a center for the planned experiment was adequate (Òable 1).

The maximalization of the target function (2) was done by means of a gradient optimization procedure (Eureka 2000). The coordinates of the optimal working point for the process with reference to the nutrient medium components were as follows:

Y_{mod}^{max} =10.53, for X_1= +1 (fructose – 30 g.dm^{-3}); X_2= -1 (yeast extract – 5 g.dm^{-3}); X_3= -0.645 (meat peptone – 8.71 g.dm^{-3}) and X_4= 0 (KH_2PO_4 – 0.2 mmol) (3)

Table 4

Factor	Coded value		
	-1	0	1
X_1-fructose, g.dm^{-3}	25	35	45
X_2-yeast extract, g.dm^{-3}	3	5	7
X_3-meat peptone, g.dm^{-3}	7	9	11
X_4-KH_2PO_4, mmol	Fixed at 0.2 for all		

The graphic of the dependence of phytase activity on the changes of X_1 and X_2, (Table 1) and the optimal values of X_3 and X_4 in (3) is shown on Fig. 1.

The proofing of the hypothesis about the equality of the mathematical expectations with regard to the results of the experiment in extreme conditions with

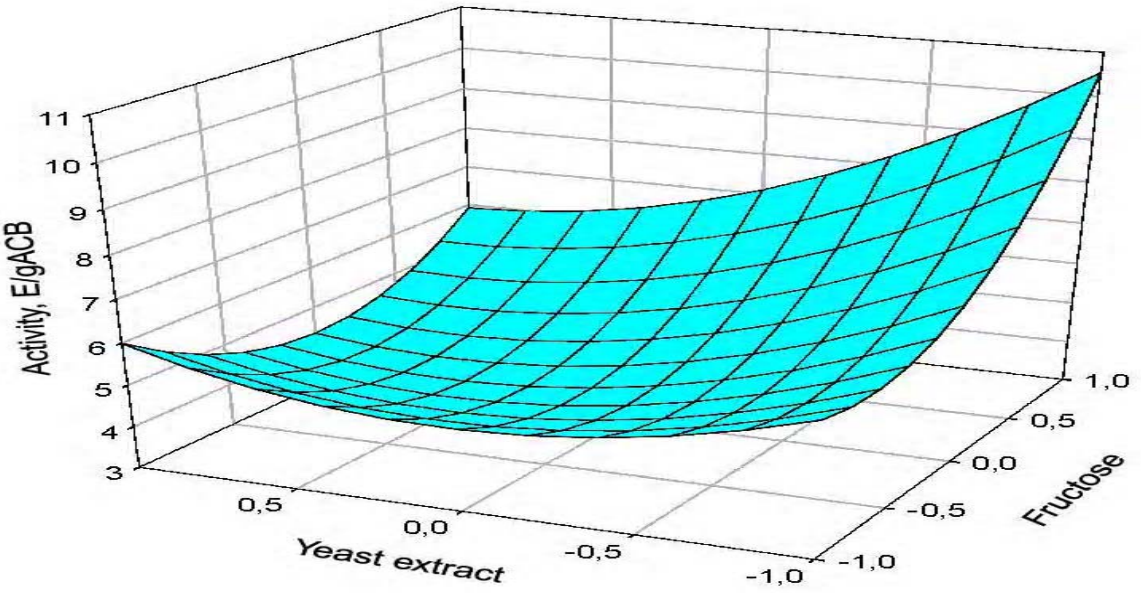

Fig. 1. Y_{mod}=f(X_1,X_2) at optimal value of X_3= -0.645 and X_4= 0

Table 5

Experiment №	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Y^*_{exp}	4.17	3.36	7.77	3.3	2.42	2.65	4.27	4.17	4.42	2.95	3.72	3.53	3.71	2.93	3.53
Y^*_{mod}	3.55	4.03	7.1	3.92	2.51	2.51	4.24	4.24	4.33	4.33	2.98	2.98	3.39	3.39	3.39
Significance F*	0.0233														

that predicted in (3) was checked according to Mason et al. (2003). The following results were obtained:

$$Y^{max}_{exp,1}=9.93;\quad Y^{max}_{exp,2}=9.98;\quad Y^{max}_{exp,3}=10.20;$$
$$Y^{max}_{exp,4}=10.80;\quad Y^{max}_{exp,5}=10.96;\quad Y^{max}_{exp,6}=10.90;$$
$$\bar{Y}_{exp}=10.46;\ S_x=0.477,\ n=6,\qquad |t_{calc.}|=0.349.$$

With regard to $\nu=5$ and $\alpha=0.05$, $t_{crit.}=2.571$ (Student's table), the condition $|t_{calc.}|<t_{crit.}$ was fulfilled and the hypothesis accepted – there was no statistically significant difference between the predicted and experimentally reported values of phytase activity on the extreme level.

The enzyme activity of *Candida melibiosica* 2491 in the optimized nutrient medium was 22.5% higher than in the primary one.

The information obtained is a prerequisite for a new series of experiments in a working point (*) with real and coded values of the variables, shown in Table 4.

The observations were performed according to the matrix of the Box-Behnken plan for 3 factors and 3 variation levels (Mason et al., 2003). The following results were obtained (Table 5) with an adequate mathematical model Y^*_{mod} - (4):

$$Y^*_{mod}=3.39+0.864.X_1-0.675.X_2-0.915.X_1.X_2+0.491.X_1^2+0.769.X_2^2-0.504.X_3^2 \quad (4)$$

The following conclusions are based on this information:

- from a mathematical point of view, Y^*_{mod} was substantially different from (2) in terms of quality and quantity;

- the analytical studies of (4) did not lead to any considerable increase of the enzyme activity of *Candida melibiosica* 2491. In these conditions, the overexpenditure of the carbon source was not justified economically. The higher fructose concentration probably lead to repression, which reflected on the physiological and biosynthetic behavior of the strain;

- trial reproduction (Cochran's criterion) in optimal conditions for the medium (*) was not achieved.

Conclusion

- A new nutrient medium composition for phytase biosynthesis of *Candida melibiosica* 2491 was obtained on the basis of an optimal composite plan and regulated variation range of independent variables with the methods of mathematical modeling, namely: fructose – 30 g.dm^{-3}, yeast extract – 5 g.dm^{-3}, meat peptone – 8.71 g.dm^{-3} and KH_2PO_4 – 0.2 mmol. Phytase activity increased with 22.5% in the optimized medium.

- The results in other experimental conditions were economically unjustifiable and had a purely mathematical (formal) importance.

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