Bulgarian Journal of Agricultural Science, 20 (Supplement 1) 2014, 87–92
Agricultural Academy

OPTIMIZATION OF NUTRIENT MEDIUM COMPOSITION BY MATHEMATICAL MODELING FOR PRODUCTION OF *RHIZOPUS ARRHIZUS* KB-2 LIPASE

M. YORDANOVA[1*], S. ILIEVA[1], B. GORANOV[2], Y. EVSTATIEVA[1], K. MILANOVA[1], K. BOZHANCHEV[1], R. DENKOVA[1] and D. NIKOLOVA[1]
[1] *Sofia University "St. Kliment Ohridski", Department of Biotechnology, Faculty of Biology, BG – 1164 Sofia, Bulgaria*
[2] *University of Food Technologies, Department of Microbiology, BG – 4000 Plovdiv, Bulgaria*

Abstract

YORDANOVA, M., S. ILIEVA, B. GORANOV, Y. EVSTATIEVA, K. MILANOVA, K. BOZHANCHEV, R. DENKOVA and D. NIKOLOVA, 2014. Optimization of nutrient medium composition by mathematical modeling for production of *Rhizopus arrhizus* KB-2 lipase. *Bulg. J. Agric. Sci.,* Supplement 1*:* 87–92

Lipases are enzymes that hydrolyze mono-, di- and tri-acylglycerols present in the oil-water interface. Lipases have potential application in different industrial sectors such as waste water treatment, cosmetics, detergents and in the fuel sector. Among all lipase producing organisms, fungi are considered to be an efficient source of lipase for industrial applications due to the production of extracellular enzymes and easy separation of fermentation medium. Fungi from *Rhizopus* sp. are important producers of lipases especially suited for interesterification of fats and oils in both the food and pharmaceutical manufactures. In the last decades, various methods were employed to optimize fermentation conditions to enhance the lipase production, especially in designing media composition by mathematical modeling. Predictive models have been accepted as informative tools for rapid and cost effective study of microbial products development and scientific purposes. Response surface methodology (RSM) especially is a mathematical modelling system, which assesses the relationships between the response(s) and the independent variables. Central composite design (CCD) is an experimental design, useful in response surface methodology in order to define the importance of the independent variables, alone or in combination, in the model. The current work proposes a simple mathematical model which relates the production of lipase with the concentration of different nutritive compounds used in fermentation medium.

In this context, the influence of some carbon, nitrogen and phosphorus sources on lipase biosynthesis by the fungal strain *Rhizopus arrhizus* KB-2 during batch cultivation process was investigated in the present study. According to our previous experiments with a different nutritive compounds in the fermentation medium glucose, soy flour and yeast extract have a significant effect on the yield of lipase. Variation ranges of these components were also determined during individual cultivation processes. For optimization of the medium composition for extracellular lipase production by *Rhizopus arrhizus* KB-2 in submerged fermentation central composite design of type 2^3 was used in order to determine combined influence of the nutrients. It was found that glucose has to be in small amounts (4.3 $g.dm^{-3}$) until soy flour and yeast extract have optimal values of 28.5 $g.dm^{-3}$ and 9.3 $g.dm^{-3}$ in the investigated factor space. Lipase activity of 60.72 FIP $U.cm^{-3}$ was detected experimentally after optimization of the nutrient medium composition by the investigated mathematical model. Based on the results obtained central composite design is suitable for optimization of medium composition for lipase production by the fungal strain *Rhizopus arrhizus* KB-2 during batch cultivation and significant enzyme activity was reached by using of minimum types of nutritive sources.

Key words: Rhizopus, lipase, mathematical modeling

**E-mail: maria_jord@mail.bg*

Introduction

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) are enzymes that catalyze a variety of reactions such as hydrolysis, transesterification and ester synthesis (Dalmou et al., 2000). Owing to their catalytic versatility, these hydrolases have received considerable attention with a view to biotechnological applications in organic chemical processing, detergent formulations, synthesis of biosurfactants, the oleochemical, dairy industry, paper manufacture, nutrition, cosmetics and pharmaceutical processing (Ghosh et al., 1996; Sharma et al., 2001). Filamentous fungi have a great potential to produce extracellular lipases, which are generally used as purified enzymes (Pandey et al., 1999). Particularly, the lipase from *Rhizopus* has 1,3-regioselectivity and can selectively catalyze the hydrolysis of triacylglycerols to produce some specific industrial products (Dobrev et al., 2011). Various methods were employed to optimize the fermentation process to enhance production of lipase. When developing industrial fermentation, designing media and optimizing fermentation conditions are of critical importance because these factors could strongly interfere with the yield of lipase production (Muralidhar et al., 2001; Elibol et al., 2002). Experimental design techniques present a more balanced alternative to the one-factor-at-a-time approach for fermentation improvement (Gochev et al., 2012). Mathematical models provide a strategy for solving problems in industrial fermentation processes (Rajendran et al., 2009).

In this context, the aim of the present study was to apply mathematical modeling for enhancement of the extracellular lipase production by *Rhizopus arrhizus* KB-2 through nutritive medium engineering. For optimization of the medium composition central composite design of type 2^3 was used in order to determine combined influence of the nutrients.

Materials and Methods

Microorganism and culture maintenance

Rhizopus arrhizus KB-2 was obtained from microbial type culture collection of Department of Biotechnology, Faculty of Biology, Sofia University "St. Kliment Ohridski". Stock cultures of the investigated strain were maintained on potato dextrose agar at 28–30ºC for 5–7 days.

Fermentation conditions for lipase production

The batch nutrient medium used for investigation of lipase production by the fungal strain *Rhizopus arrhizus* KB-2 was described by Li et al. (2006) and had the following composition ($g.dm^{-3}$) with some modifications: glucose, 5; soybean flour, 30; yeast extract, 10; K_2HPO_4, 5; $MgSO_4$, 1; $(NH_4)_2SO_4$, 2.

Biosynthesis of lipase by the strain *Rhizopus arrhizus* KB-2 was carried out in 500-ml Erlenmeyer flasks containing different concentrations of glucose (0–20 g/dm^3), soybean flour (0–40 g/dm^3) and yeast extract (0–20 g/dm^3). Flasks were incubated with stirring (250 rpm) at 28–30ºC for 72 h. After fermentation, the culture was filtered, and the filtrate was used for the analysis of lipase activity.

Lipase activity assay

Lipase activity was estimated with olive oil emulsion using the procedure of the Fungi Lipase-International F.I.P. Standard (ETA, 2002). One unit of enzyme activity (FIP Unit) is defined as that quantity of lipase that liberates the equivalent of 1μmole of fatty acid from olive oil per minute at 35ºC and pH 7.0.

Experimental design

Response surface methodology (RSM) offers a large amount of information from a small number of experiments because of using special designs those help the appropriate model be fitted to the response(s). A central composite design (CCD) includes three groups of design points. Factorial points that consists of all possible combinations of the +1 and -1 levels of the factors; star or axial points that have all of the factors set to 0, the midpoint, except one factor, which has the value +/– Alpha; and center points, which are points with all levels set to coded level 0, the midpoint of each factor range. This gives an adequate estimate of the variation of the response and provides the number of degrees of freedom needed for an adequate statistical test of the model. To summarize, central composite rotatable designs require 5 levels of each factor: –Alpha, –1, 0, 1, and +Alpha. Central Composite Design (CCD) was used for optimization of the composition of the nutritive medium. The mathematical processing of the results from the experiments was accomplished using Microsoft Excel and Stat graphics Centurion XV (trial version). The value of the coefficients in equation 2 was calculated in accordance with the dependence:

$$(1) \quad b_{ji} = \sum_{i}^{N} X_{ji} Y_i \Big/ \sum_{i}^{N} X_{ji}^2 ;$$

$$(2) \quad b_{ji} = \sum_{i}^{N} X_j' Y_i \Big/ \sum_{i}^{N} (X')_j^2; \; X_j' = X_j^2 - \left(\sum_{i=1}^{N} X_{ji}^2 \Big/ N \right),$$

where X_{ji} is the coded value of the experimental factor (I = 1, 2, 3, 4; j = 1, 2, 3, 4);

Y_i is the observed value of the test function (lipase activity) in the corresponding point;

N is the number of experimental factors.

The optimization of the target function was performed by the gradient method, with fixing of some factors of certain level. The desirability function for optimization *d(y)* expresses the desirability of a response value equal to *y* on a scale of 0 to 1. This function takes one of three forms depending on whether the response is to be maximized, minimized, or a target value hit.

Results and Discussion

Lipase activity and production depend upon the composition of the fermentation medium. According to our previous results glucose, soy flour and yeast extract in the nutritive medium strongly interfere the lipase activity of *Rhizopus arrhizus* KB-2 during batch fermentation process. Based on the results from a single factor analysis, the investigated nutrient sources have a positive effect on the lipase production in concentrations up to 10 g.dm^{-3} glucose, 30 g.dm^{-3} soy flour and 15 g.dm^{-3} yeast extract during batch fermentation processes. Lipase activity of 46.17 FIP U.cm^{-3} was detected at 72th h of the cultivation using these concentrations of nutrient sources. Mathematical-statistic methods for modeling and optimization allow to determinate the optimal nutritive medium composition with a few experiments and to estimate the combined effects of different nutritive medium components. For optimization of the nutritive medium composition for extracellular lipase production by *Rhizopus arrhizus* KB-2 in submerged fermentation CCD of type 2^3 with star arm was used. Such models can be obtained by CCD (Equation 3).

$$(3) \quad Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{1<j<i<k}^{k} b_{ij} X_i X_j + \sum_{i=1}^{k} b_{ij} X_i^2 .$$

The major advantage of this method is the opportunities for estimation of the inhibitory and stimulating effect of influencing factors. Based on the results obtained from single factor experiments, the factor area and the limits of variation of nutritive medium components were determined and shown on Table 1.

The rest of the nutritive medium components were fixed at constant levels. CCD was not orthogonal. It was reduced

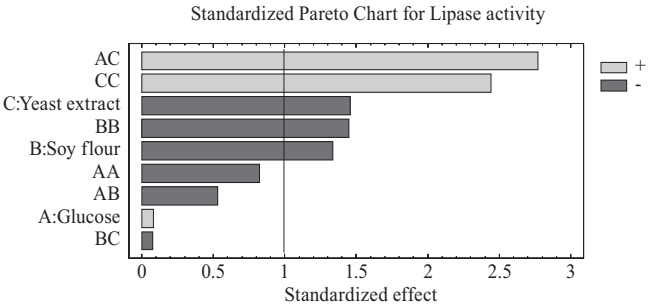

Fig. 1. Pareto-diagram for the significance of the coefficients in the mathematic model

to orthogonal by selecting an appropriate star arm of size α. The value of the star arm depends on the number of input factors (n = 3). The size of the star arm $\pm\alpha$ = 1.41421 was determined. The experimental design and the results for production of extracellular lipase by *Rhizopus arrhizus* KB-2 in batch fermentation are shown in Table 2.

On the basis of the results obtained mathematical-statistic analysis of the CCD was carried out and regression coefficients were calculated and presented on Table 3.

This pane displays the regression equation which has been fitted to the data. The equation of the fitted model is:

Lipase activity = 15.78 – 3.85*Soy flour – 4.2*Yeast extract + 9.78*Glucose*Yeast extract – 5.10*Soy flour^2 + 8.61*Yeast extract^2,

where the values of the variables are specified in their original units. The results of the analysis of the proposed mathematical model are shown in Table 4. In accordance with the data in Table 4 and Pareto – diagram (Figure 1) all the insignificant factors were excluded from the model.

The ANOVA table partitions the variability in lipase activity into separate parts for each of the effects. It then tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. In the present study, 5 effects had P-values less than 0.05, indicating that they were significantly different from zero at the 95.0% confidence level. The R-squared statistic indicated that the model, as fitted, explained 98.6251% of the variability in lipase activity. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables,

Table 1

Limits of variation of nutritive medium components

Factor	Compound, g.dm^{-3}	–α (–1.41421)	–1	0	+1	+α (+1.41421)
A	Glucose	4.3	6	10	14	15.7
B	Soybean flour	24.3	26	30	34	35.7
C	Yeast extract	9.3	11	15	19	20.7

Table 2
Experimental design and experimental results for modeling of extracellular lipase production by *Rhizopus arrhizus* KB-2 in batch fermentation

Row	Factors (coded value)			Factors, g/dm^3			Observed Value	Fitted Value	Lower 95.0% CL for Mean	Upper 95.0% CL for Mean
	Glucose	Soy flour	Yeast extract	Glucose	Soy flour	Yeast extract				
1	0	0	0	10	30	15	15.78	15.78	8.103	23.461
2	0	0	0	10	30	15	14.165	15.78	8.103	23.461
3	0	0	0	10	30	15	12.25	15.78	8.103	23.461
4	0	0	0	10	30	15	21.3	15.78	8.103	23.461
5	–1	–1	–1	6	26	11	39.35	37.11	25.599	48.637
6	1	–1	–1	14	26	11	15.665	17.56	6.045	29.083
7	–1	1	–1	6	34	11	18.05	29.41	17.900	40.937
8	1	1	–1	14	34	11	6.0	9.86	–1.653	21.384
9	–1	–1	1	6	26	19	4.17	9.16	–2.356	20.680
10	1	–1	1	14	26	19	38.75	28.71	17.196	40.234
11	–1	1	1	6	34	19	0.935	1.46	–10.056	12.981
12	1	1	1	14	34	19	8.835	21.01	9.497	32.535
13	–1.41421	0	0	4.3	30	15	16.98	15.78	8.103	23.461
14	1.41421	0	0	15.7	30	15	14.22	15.78	8.103	23.461
15	0	–1.41421	0	10	24.3	15	4.88	11.01	–2.283	24.318
16	0	1.41421	0	10	35.7	15	17.55	0.128	–13.172	13.429
17	0	0	–1.41421	10	30	9.3	47.15	38.94	25.648	52.250
18	0	0	1.41421	10	30	20.7	30.15	27.06	13.7654	40.367

Table 3
Regression coefficients for lipase activity

Coefficient	Estimate
constant	15.7825
B:Soy flour	–3.84975
C:Yeast extract	–4.20139
AC	9.77688
BB	–5.10484
CC	8.61273

was 95.5522%. The standard error of the estimate showed the standard deviation of the residuals to be 3.63322. The mean absolute error (MAE) of 2.29851 is the average value of the residuals. The Durbin-Watson (DW) statistic tests the residuals to determine if there is any significant correlation based on the order in which they occur in the data file. Since the P-value is greater than 5.0%, there was no indication of serial autocorrelation in the residuals at the 5.0% significance level.

Graphical interpretations of the proposed mathematical model are shown in Figure 2.

Table 4
Analysis of variance for lipase activity

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value	Signiicance
A:Glucose	0.672977	1	0.672977	0.01	0.9365	Insignificant
B:Soy flour	177.847	1	177.847	1.79	0.2183	Significant
C:Yeast extract	211.82	1	211.82	2.13	0.1829	Significant
AA	67.8523	1	67.8523	0.68	0.4331	Insignificant
AB	28.294	1	28.294	0.28	0.6086	Insignificant
AC	764.698	1	764.698	7.68	0.0243	Significant
BB	208.473	1	208.473	2.09	0.1860	Significant
BC	0.596778	1	0.596778	0.01	0.9402	Insignificant
CC	593.431	1	593.431	5.96	0.0405	Significant
Total error	796.974	8	99.6217			
Total (corr.)	2850.65	17				

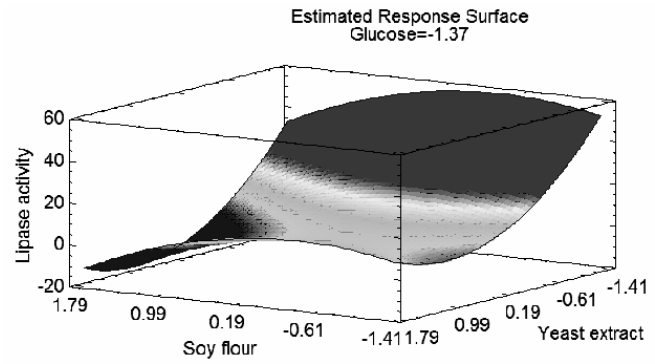

Fig. 2. Estimated response surface for influence of yeast extract and soy flour on the lipase activity (FIP U.cm^{-3}) at glucose = –1,37 (factors were presented with coded values)

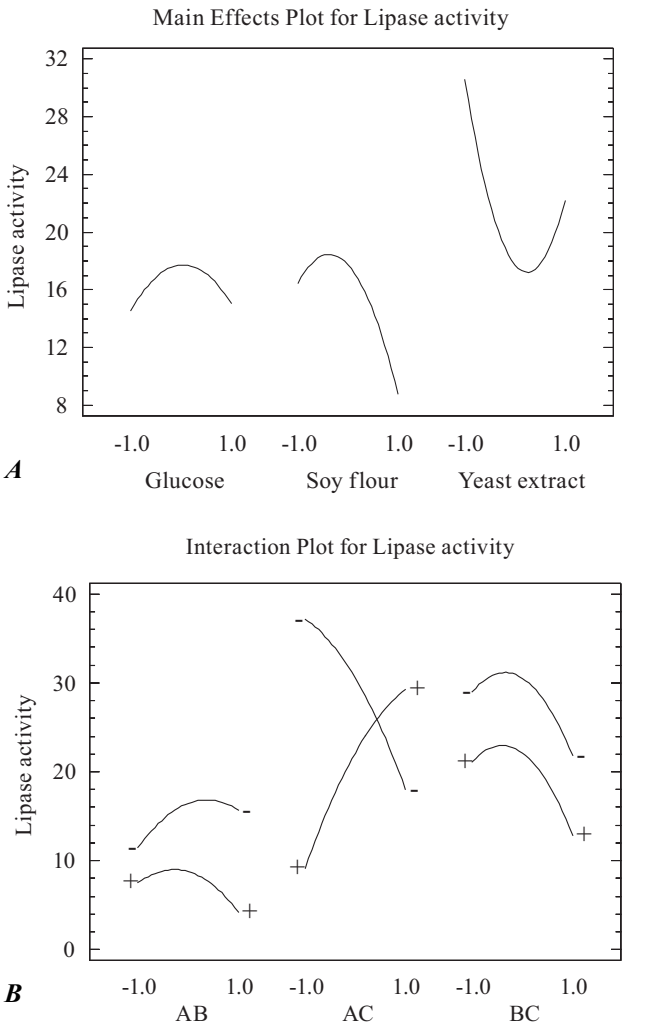

Fig. 3. Influence of the studied factor, separately (*A*) and in combinations (*B*), on the lipase activity (U.cm^{-3})

Based on the mathematical statistic analysis of the results obtained the optimal value of the target function (lipase activity) was determined as 58.556 FIP U.cm^{-3} and should be obtained during batch cultivation of the fungal strain *Rhizopus arrhizus* KB-2. The optimal concentrations of nutritive components are presented on Table 5.

The degree of influence of the studied factors separately and in combinations is presented on Figure 3.

Based on the obtained model glucose has to be in small amounts (4.3 g.dm^{-3}) until soy flour and yeast extract have optimal values of 28.5 g.dm^{-3} and 9.3 g.dm^{-3} in the investigated factor space. Lipase activity of 60.72 FIP U.cm^{-3} by fungal strain *Rhizopus arrhizus* KB-2 was detected experimentally at 72th h of the cultivation after optimization of the nutrient medium composition by the investigated mathematical model. According to the results the mathematical model fits to the experimental data obtained.

Table 5

Optimal values of glucose, soy flour and yeast extract concentrations used for enhancement of lipase production by the fungal strain *Rhizopus arrhizus* KB-2

Factor	Low	High	Optimum	Real data, g.dm^{-3}
Glucose	–1.41421	1.41421	–1.36573	4.3
Soy flour	–1.41421	1.41421	–0.353066	28.5
Yeast extract	–1.41421	1.41421	–1.41421	9.3

Conclusions

According to the results concentrations of glucose, soy flour and yeast extract influence the extracellular lipase production by the fungal strain *Rhizopus arrhizus* KB-2. As a result of the applied mathematical modeling and optimization procedure the optimal nutritive medium composition was determined (g.dm^{-3}): glucose, 4.3; soybean flour, 28.5; yeast extract, 9.3; K_2HPO_4, 5; $MgSO_4$, 1; $(NH_4)_2SO_4$, 2. Based on the results obtained central composite design is suitable for optimization of medium composition for lipase production by filamentous fungi during batch cultivation and significant enzyme activity was reached by using of minimum types of nutritive sources.

Acknowledgments

This work was supported by grant No 112 of Sofia university Science fund and grant DMU 03/12 of National Science Fund, Bulgaria.

References

Dalmou, E., J. M. Montesinos, M. Lotti and C. Casas, 2000. Effect of different carbon sources on lipase production by *Candida rugosa. Enzyme Microb. Technol*., **26:** 657–663.

Dobrev, G., B. Zhekova, P. Nedelcheva, R. Chochkov and A. Krastanov, 2011. Characterization of crude lipase from *Rhizopus arrhizus* and purification of multiplicity forms of the enzyme. *Biotechnol. & Biotechnol. Eq*., **25** (1): 2295–2300.

Elibol, M. and D. Ozer, 2002 .Response surface analysis of lipase production by freely suspended *Rhizopus arrhizus*. *Process Biochemistry,* **38**: 367–372.

Enzyme Technical Association, 2002. GRAS notification – exemption claim for lipase enzyme preparation produced by *Aspergllus niger* that is the subject of a GRAS affirmation petition, GRASP 3G00162002.

Ghosh, P. K., R. K. Saxena, R. Gupta, R. P. Yadav and S. Davidson, 1996. Microbial lipases: production and applications. *Science Progress,* **79** (2): 119–157.

Gochev, V., G. Montero, G. Kostov, L. Toscano, M. Stoytcheva, A. Krastanov and A. Georgieva, 2012. Nutritive medium engineering enhanced production of extracellular lipase by *Trichoderma longibrachiatum*. *Biotechnol. & Biotechnol. Eq*., **26** (2): 2875-2882.

Muralidhar, R. V., R. R. Chirumamila, R. Marchant, R. and P. Nigam, 2001. A response surface approach for the comparison of lipase production by *Candida cylindracea* using two different carbon sources. *Biochemical Engineering Journal,* **9**: 17–23.

Pandey, A., S. Benjamin, C. R. Soccol, P. Nigam, N. Krieger and V. T. Soccol, 1999. The real of microbial lipases in biotechnology. *Appl Biochem Biotechnol.,* **29:** 119–31.

Rajendran, A. and V. Thangavelu, 2009. Statistical experimental design for evaluation of medium components for lipase production by *Rhizopus arrhizus* MTCC 2233. *LWT – Food Science and Technology,* **42:** 985–992.

Sharma, R., Y. Chisti and U. C. Banerjee, 2001. Production, purification, characterization and applications of lipases. *Biotechnol Adv.,* **19** (8): 627–62.