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Application of Response Surface Methodology for optimization of permeabilization process of baker's yeast

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Permeabilization was used for the purpose of transforming the cells of microorganisms into biocatalysts with an enhanced enzyme activity. Baker's yeast cells were permeabilized with various organic solvents. A high degree of catalase activity was observed upon permeabilization with acetone, chloroform, isopropyl alcohol and ethyl acetate. Response surface methodology was used to model the effect of concentration of isopropyl alcohol, temperature and treatment time on the permeabilization of baker's yeast cells to maximize the decomposition of H_2O_2. The optimum operating conditions for permeabilization were observed at 53.7% concentration of isopropyl alcohol, treatment time of 40 min and temperature of 15.6°C. A maximum value of catalase activity was found to be 6.188 U/g wet wt. and was ca. 60 times higher than the catalytic activity of yeast not treated by the permeabilization process.

Keywords: permeabilization, baker's yeast, response surface methodology, isopropyl alcohol.

INTRODUCTION

Catalase is one of the most industrially significant enzyme in view of the ability to degrade H_2O_2 into water and oxygen. It is used extensively in textile, food industry and in pharmaceutical formulations for the removal of residual hydrogen peroxide. Isolated, purified and soluble catalase preparations from animal and microbial sources are not readily recoverable from mixture for reuse. Furthermore they may also be denatured by shear forces generated during mixing or by foaming in the aerated solution. Thus making their use relatively expensive. The use of the whole yeast cells as biocatalysts is a very promising alternative and has gained a lot of interest in recent years[6, 7]. Baker's yeast are a rich source of catalase[3], unfortunately the permeability of its cell membrane is relatively low[8, 9]. Modifying cell membrane can facilitate the diffusion of substrates and products. These permeabilized cells can be reused without too much increase in the cost of recovery. The permeability of the cytoplasmic membranes of yeast cells may be improved by altering its components with various agents like organic solvents, detergents, salt, enzymes and chemicals[6, 10–15]. Specifically, this relates to the lipid fraction, which is the structural framework of cell membranes. A suitably selected substance is expected to increase the cell membrane pores, and not deactivate the intracellular enzyme. Though a number of groups have standardised permeabilization of bakers yeast with various reagents[6, 11–15], still each batch of yeast needs to standardised for optimum efficiency.

Previous research works on the subject of permeabilization are usually based on a conventional method which involves changing one independent variable while maintaining all others unchanged at a fixed level[14]. These practices are very expensive and time-consuming, therefore in this paper Response Surface Methodology (RSM) was applied for successful development, improvements and optimization of processes[16–19]. It is the objective of RSM to comprehend the relationships between changes in responses to the adjustment of design variables[20]. So, in the RSM operations algorithm, those variables which have a significant effect on the process are tested simultaneously in a minimum number, according to a suitably selected plan of experiments. This study aims to model the permeabilization process parameters of baker's yeast by using RSM, to maximize the degradation of H_2O_2 by catalase.

EXPERIMENTAL

Material and methods

One and the same batch of compressed baker's yeast, obtained locally, was stored and used within 1 week. The organic solvent used for permeabilization (acetone, benzene, chloroform, isopropyl alcohol, n-hexane, ethyl acetate, toluene), hydrogen peroxide and other chemicals were purchased from POCH S.A. (Polish Chemicals Reagents).

Permeabilization of whole yeast cells

One gram (wet wt.) of yeast cells was suspended in 20 g of phosphate buffer (pH 7.0) with organic solvents as a permeabilizing agent. The permeabilizing effect of different concentration (2–60%) of organic solvents was studied. The contents were mixed and incubated for 30 min, under shaking conditions. The treated cells were centrifuged and evaluated for the activity of catalase. The supernatant was also examined for catalase activity.

Enzyme assay

Catalytic activity was assayed by measuring the increase in dissolved oxygen resulting from the enzymatic decomposition of hydrogen peroxide, similar to the method described by Delrio et al[21]. The assays were carried out at 20°C in a jacketed vessel with a total reaction volume of 100 ml H_2O_2 solution with phosphate buffer (pH 7). Nitrogen gas was used for the desorption of the oxygen which was dissolved in the solution.

After an addition of appropriate volume of the suspension of permeabilized yeast cells the reaction was started. Then any change in the degree of saturation with oxygen was measured using an oxygen meter. The catalase activity was calculated from the slope of the oxygen concentration versus time profile after baseline

subtraction. One unit of enzyme activity is defined as that quantity which degrades 1 mmol of H_2O_2 per min under standard conditions.

Experimental design and statistical analysis

The experiments were conducted according to Central Composite Rotatable Design (CCRD) with three independent variables at five levels (–1.682, –1, 0, 1, 1.682) each (Table 1). The variables were: temperature (X_1), concentration of isopropyl alcohol (X_2) and time (X_3) of permeabilization process for the catalase activity – dependent variable (A). The study included 20 experiments with 6 runs at central level as replicates (Table 2).

Table 1. Values of independent variables at different levels of the CCRD

Coded value (level)	Real value of variables		
	Temperature [°C]	Concentration [%]	Time [min]
–1.682	6.6	7	7
–1	10	20	20
0	15	40	40
1	20	60	60
1.682	23.4	73	73

Table 2. Experimental design of coded values

Run	Temperature x_1	Concentration x_2	Time x_3	Enzyme activity [U/g]
1	–1	–1	–1	271.0
2	–1	1	–1	4582.4
3	–1	–1	1	1873.4
4	–1	1	1	5056.2
5	1	–1	–1	2708.4
6	1	1	–1	4951.8
7	1	–1	1	3687.0
8	1	1	1	3559.2
9	1.682	0	0	2549.0
10	–1.682	0	0	349.0
11	0	1.682	0	4277.1
12	0	–1.682	0	120.0
13	0	0	1.682	5100.4
14	0	0	–1.682	5227.5
15	0	0	0	5904.0
16	0	0	0	5748.2
17	0	0	0	5879.4
18	0	0	0	5784.0
19	0	0	0	5804.0
20	0	0	0	5765.0

A second order quadratic model Eq. (1) was then fitted to the data by the multiple regression procedure:

$$A = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j \qquad (1)$$

Where b_0 is the value of fitted response at the centre point of design, i.e. point (0, 0, 0). The model permitted an evaluation of linear b_i, quadratic b_{ii} and interactive terms b_{ij} of the independent variables on the dependent variable. Statistical analysis of the mathematical model was performed using ANOVA (Analysis of Variance). The F test was employed to evaluate the statistical significance of the quadratic polynomial. The R^2 statistic indicates the percentage of the variability of the optimization parameters, which is explained by the model. The response surface plots were used for determining the optimum level of the significant variables for maximal catalase activity during the decomposition of hydrogen peroxide.

Storage stability

The ability of permeabilized cells (treated with isopropyl alcohol) to retain catalase during storage was studied. A suspension of 1 g of cells in 20 ml potassium buffer (pH 7.0) was stored at 4°C. At regular intervals a predetermined volume of suspension was separated by centrifugation and catalase activity was determined in the separated cells.

RESULTS AND DISCUSSION

Whole cells of yeast exhibited very low catalase activity (100 U/g wet wt.). The permeabilization of baker's yeast was carried out by using various organic solvents (acetone, benzene, chloroform, isopropyl alcohol, n- hexane, ethyl acetate, toluene) for catalase activity (Fig. 1).

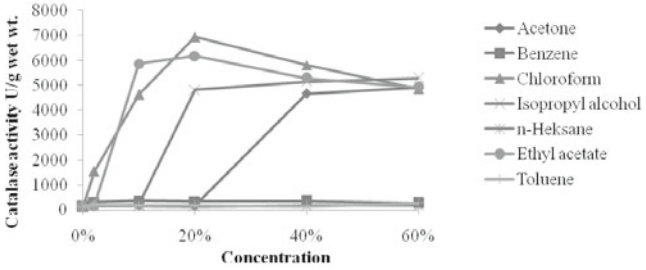

Figure 1. Effect of varying concentrations of permeabilizing agents at 20°C for 30 min

A comparative study of their efficiency revealed that ethyl acetate, chloroform and isopropyl alcohol could permeabilize the yeast cells most efficiently amongst them. However, the first two solvents are toxic and irritant. Therefore, study of the optimal condition of the permeabilization process was performed with isopropyl alcohol. Our earlier studies[22] and other researchers[12, 14] reported that permeabilization efficiency is impacted by the concentration of the permeabilizing agent, temperature and treatment time.

Optimization of permeabilization of yeast cells

Response Surface Methodology has been used to evaluate the relation between the experimental and observed results, also to optimize the significant variables (temperature, concentration of isopropyl alcohol, and time) for the permeabilization of yeast. Table 2 shows the matrix design of the coded independent variables and the experimental results according to the above 3^2 full factorial design.

The three-dimensional response surfaces for catalase activity: temperature, concentration of isopropyl alcohol and time duration were plotted (Fig. 2) and showed a non-linear parabolic relationship between the independent variables. Figure 2a shows the effects of temperature, concentration of isopropyl alcohol and their mutual interaction on catalase activity. Permeabilization with low concentration of alcohol and low temperature of process showed the lowest enzyme activity. The catalase activity

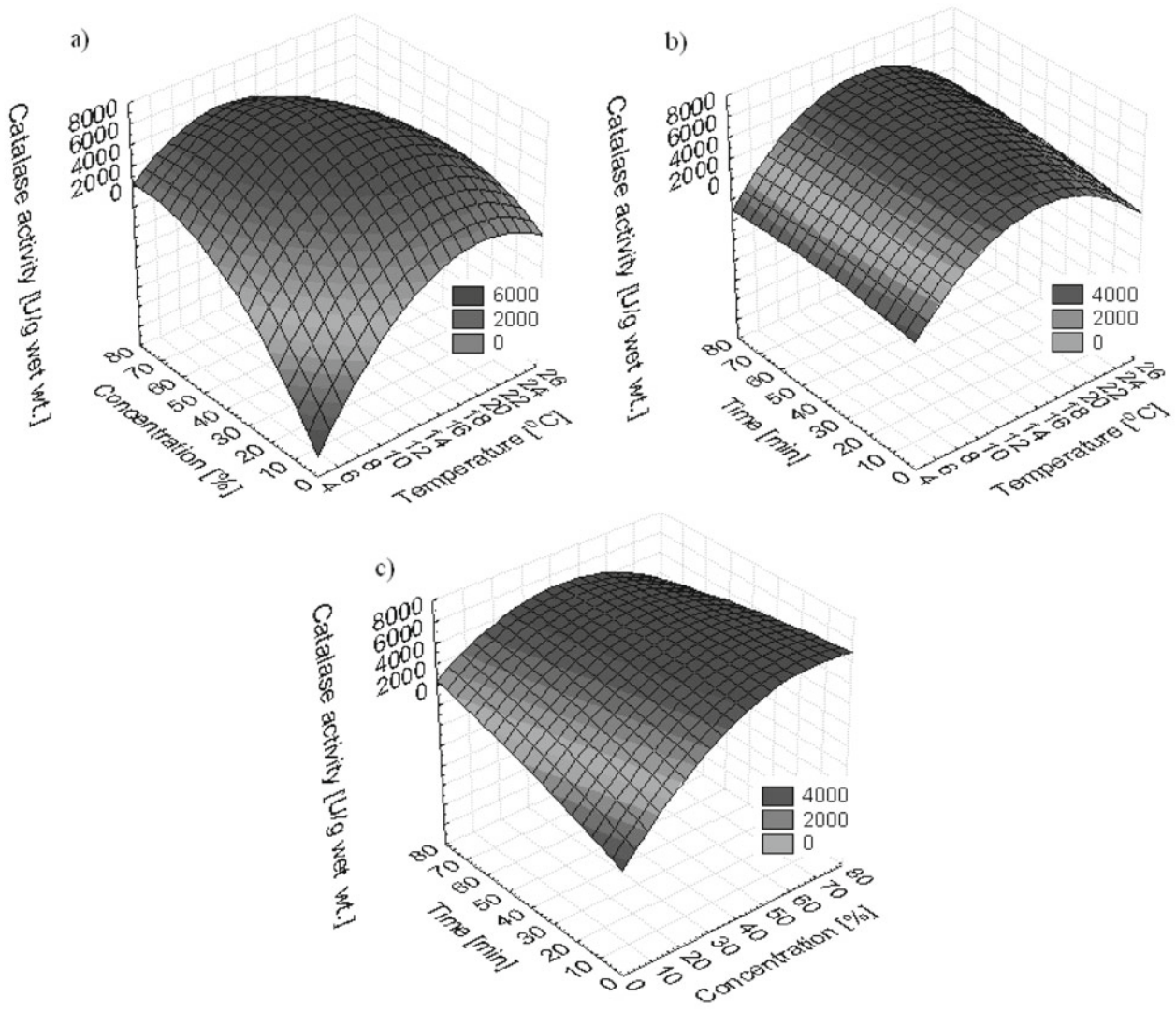

Figure 2. Response surface plot representing the effect of a) concentration of isopropyl alcohol and temperature, b) temperature and time, c) concentration of isopropyl alcohol and time on catalase activity

increased with the increases of temperature up to ca. 15°C and concentration of alcohol up to ca. 30% and thereafter decreased with further increases to 26°C and 60%. This may be due to the deactivation of enzymes at higher temperatures or higher concentration of the solvent. Figure 2b depicts the response surface plot as a function of temperature versus time. Change of time does not significantly affect the curvature of the surface. From a graphical representation of the dependence of catalase activity on the concentration of permeabilizing agent and time (Fig. 2c) we can observe high permeabilization effectiveness within the range of concentration 45–55%, while below and above these ranges a significant decrease of activity can be noticed. It is also clear that the effect of concentration on catalase activity is more important than the time. Unfortunately, very high concentration of alcohol caused deactivation of enzyme. Therefore the enzyme activity increased with increase of concentration up to 50% and thereafter decreased with further decrease to 80%.

The second order polynomial equation was fitted to the experimental data. The results of the estimated coefficients of Eq. (1) together with their statistical assessment at statistically significant >95% confidence level are presented in Table 3. Values of the coefficients in mathematical model for each variable are given together with the result of the t-Student test at the freedom degree equal to 10. The linear, quadratic and interaction coefficients (b_1, b_2, b_{11}, b_{22}, b_{12} and b_{23}) were found significant ($p < 0.05$). This suggested that the concentration of isopropyl alcohol and temperature has a direct relationship with the permeabilization of yeast cells process. This result confirms earlier research by scientists who have proven that alcohol concentration and temperature are two critical factors for the effective permeabilization of yeast cells[17].

The final estimative response model equation in terms of catalase activity after neglecting the effect of non-significant terms was:

$$A = 5795.68 + 499.61X_1 + 1215.54X_2 - 1422.09X_1^2 - 1157.15X_2^2 - 672.32X_1X_2 - 437.49X_2X_3 \quad (2)$$

The analysis of variance (ANOVA) results demonstrates that the regression is highly significant and presents good determination coefficient ($R^2 = 0.977$). The closer the R^2 is to 1, the stronger the model is and the better it predicts the response. High value of correlation coefficient ($R = 0.988$) explains an excellent correlation between the independent variables[23]. The F-test yielding a very low probability level ($F = 228$, $p = 0.0002$) revealed high statistical relevance of the regression model. The model predicted the optimal values of test factors in the

Table 3. Estimated regression coefficients

Term	Coefficient	SE coefficient	T DF = 10	p-value
β_0	5795.68	167.9245	34.5136	0.000000
β_1	499.61	111.4075	4.4846	0.001171
β_2	1215.54	111.4075	10.9107	0.000001
β_3	106.05	111.4075	0.9519	0.363586*
β_{11}	−1422.09	108.4378	−13.1144	0.000000
β_{22}	−1157.15	108.4378	−10.6711	0.000001
β_{33}	−108.99	108.4378	−1.0050	0.338573*
β_{12}	−672.32	145.5683	−4.6186	0.000953
β_{13}	−311.29	145.5683	−2.1384	0.058190*
β_{23}	−437.49	145.5683	−3.0054	0.013221

$R = 0.988$; $R^2 = 0.977$;
SE coefficient – standard error of coefficient, T – test coefficient, DF – degree of freedom.

coded units were $X_1 = -0.129$, $X_2 = 0.685$ and $X_3 = -1.058$. At these values, the temperature, concentrations of isopropyl alcohol and time duration were 15.6°C, 53.7% and 40 minutes, respectively. At these conditions of process variables the predicted value of maximum catalase activity was found to be 6,188 U/g wet wt. Permeabilization of the yeast cells has been conducted and the catalase activity has been measured at the optimal conditions. The obtained value differed from the RSM estimated value only by 5%.

Storage stability

Yeast cells after permeabilization at the optimal conditions have been tested with respect to maintaining enzymatic activity during storage. The cells showed 20% loss of catalase activity when they were stored in phosphate buffer pH 7.0 (Fig. 3) at 4°C, for a period of 7 days. The activity observed in the supernatant was very low. This may be due to inactivation of the enzyme in the medium after leakage.

The CTAB-permeabilized yeast cells which were kept under similar conditions had a half-life of intracellular catalase was 3.4 days only[12].

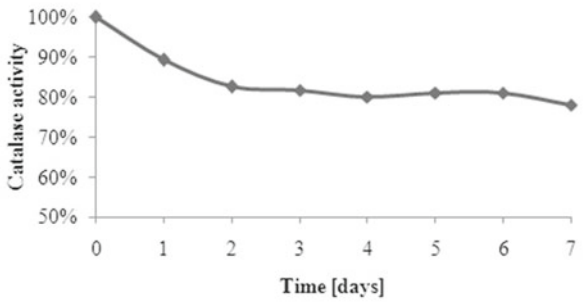

Figure 3. Storage stability of catalase from permeabilized baker's yeast cells

CONCLUSION

Isopropyl alcohol can effectively improve the permeability of cell membrane of baker's yeast. Statistical optimization of decomposition of hydrogen peroxide by intracellular catalase has been successfully carried out using RSM based on the 2^3 factorial CCRD. The proposed mathematical model with estimated parameters describes well the permeabilization process. The optimum operating conditions for the permeabilization process to achieve maximum enzyme activity were isopropyl alcohol concentration of 53.7%, 15.6°C temperature and process duration of 40 min. Under these conditions of process variables the predicted value of maximum catalase activity was found to be 6,188 U/g wet wt. The fact that isopropyl alcohol permeabilized cells retained catalase activity for a quite long period suggested that these permeabilized cells could be used as a source of catalase for different applications. Furthermore, the use of permeabilized cells can help to overcome the problems and costs associated with enzyme extraction and purification from yeast cells and in the development of a low-cost technology for decomposition of H_2O_2.

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