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EFFECT OF TEMPERATURE, CONCENTRATION OF ALCOHOLS AND TIME ON BAKER'S YEAST PERMEABILIZATION PROCESS

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A b s t r a c t

Baker's yeast beyond the traditional use in the food industry may be used to carry out biotransformations. The effectiveness of yeast as biocatalysts is based on the presence of large amounts of intracellular enzymes, whose efficiency can be repeatedly increased by permeabilization. It is the process of increasing the permeability of cell walls and membranes in order to facilitate reagents access to the intracellular enzyme. Alcohols permeabilization process allows for approx. 50-fold increase in catalase activity of baker's yeast. In this paper, the influence of physical and chemical parameters on the effectiveness of permeabilization of baker's yeast cells using alcohols was analyzed. Research has shown that with increasing temperature of permeabilization process better results are achieved using a lower alcohol concentration. Based on presented response surface graphs, we can also indicate a negligible impact of duration time on the process efficiency.

Abbreviations
T – temperature
S – concentration
t – time
RSM – response surface methodology

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Introduction

As the name suggests, baker's yeast are microorganisms used in food industry, particularly in bakery products. For thousands of years they have served as an ingredient for dough leavening. Carbon dioxide evolved due to their fermentation ability causes the dough to leaven, transforming it into bakery products with a spongy and porous structure. Apart from the fermentation processes, baker's yeast is used in intensively developing processes of food products enrichment, i.e. fortification (GONCERZEWICZ, MISIEWICZ 2011) as well as in microencapsulation of e.g. food aromas (WOJTYŚ, JANKOWSKI 2004) and vitamins (CZERNIAK, JANKOWSKI 2013). Baker's yeast, due to its high content of various intracellular enzymes, has also found an application as catalyst in bioprocesses. They are a rich source of catalase – one of the most industrially significant enzyme in view of the ability to degrade H_2O_2 into water and oxygen.

Many scientists have proven that the use of biocatalysts in the form of whole cells of microorganisms is often more effective than the use of purified enzymes (SEKHAR et al. 1999, VENKATESHWARAN et al. 1999, XU et al. 2016). However, low permeability of cell wall and membrane contributes to the slow rate of reactions catalyzed by whole cells enzymes. Such difficulties can be overcome by the application of permeabilization technique which is to improve the permeability of cell wall and membrane of microorganisms for facilitating the diffusion of reaction reagents, while also maintaining the cell's properties, including enzymatic activity and structure.

The most common technique of increasing permeability of cell membranes is chemical permeabilization with the use of detergents (CHOW, PALECEK 2004, GOUGH et al. 2001, KIPPERT 1995, PATIL et al. 2017, PRESECKI, VASIĆ-RACKI 2005) and organic solvents (KONDO et al. 2000, KUMARI et al. 2011, PANESAR et al. 2007). The literature data show that the choise of a chemical agent is dependent on the type of a permeabilized microorganism (CHOW, PALECEK 2004, GALABOVA et al. 1996). Additionally, applied agent should not affect the activity of intracellular enzymes. Therefore, in order to determine the optimal conditions of the process for selected microorganisms, it is necessary to analyze the influence of numerous various substances on their permeability, thus determining the most effective one. Chemical permeabilization of yeast carried out with the use of detergents causes considerable changes in the cell membrane structure, in comparison to organic solvents. This, in consequence, leads to cellular leakage or even to its destruction (ALAMAE, JARVISTE 1995). For this reason if the second reaction with the use of the same biocatalysts is necessary, it is not advised to obtain them by the application of permeabilization method with the use of detergents, but to select the most effective permeabilizing agent from among alcohols.

The increase in permeability of cell wall and membrane of microorganisms is determined not only by the type of permeabilizing agent but also the operating conditions. This study aims to determine the effect of temperature (T), concentration of the chemical substance (S) and treatment time (t) of the permeabilization process on its effectiveness. The intent is to determine the influence of mutual interactions of analyzed parameters on the effectiveness of the process. For this purpose the response surface methodology has been applied in the study. The effectiveness of the process has been represented by measuring intracellular catalase activity.

Materials and methods

Biological material and chemical reagents

The producer of baker's yeast (*Saccharomyces cerevisiae*) is Lasaffre bio-corporation S.A. from Wolczyn, Poland. A fresh block of moist yeast was stored in accordance with the producer's recommendations, at a temperature below 10°C. For the whole duration of the study the yeast maintained the moisture content of 68.5%. Organic solvents have been used, i.e. ethanol (ethyl alcohol), 1-propanol and 2-propanol. Catalase activity was assayed with the use of hydrogen peroxide 30%, phosphate buffer pH 7 and nitrogen gas from EuroGaz Gdynia. All the chemical reagents mentioned above (except the nitrogen) were pure for the analysis, obtained from POCh S.A. Gliwice.

Permeabilization process

1 g of baker's yeast and 20 g of a permeabilizing agent were placed into a 50 cm^3 beaker. The concentration of alcohol was according to the selected plan of experiments. With the use of a mechanical stirrer the biological material was distributed appropriately. The suspension was vigorously stirred using a water-bath with a shaking mechanism. The device enabled to conduct the permeabilization process at an appropriate temperature (according to the plan of experiments). After an appropriate amount of time, according to the plan, a sample of yeast cells was taken and their intracellular catalase activity was assayed with a method described below.

Catalase activity assay

The catalase activity was determined using an oxygen method. For this purpose the increase in dissolved oxygen resulting from the enzymatic decomposition of hydrogen peroxide was measured. A detailed description of the process was presented in previous publications (TRAWCZYŃSKA, WÓJCIK 2014, TRAWCZYŃSKA, WÓJCIK 2015).

Response surface methodology

Study on the effects of physico-chemical parameters on the effectiveness of permeabilization process of baker's yeast cells was conducted in accordance with the principle of optimization of chemical processes using response surface methodology. This method is a set of statistical and mathematical techniques used in developing, improving and optimizing processes. In the RSM algorithm

Table 1

Central composite design matrix

No	Temperature	Concentration	Time	Enzyme activity [$U \cdot g^{-1}$]		
				ethanol	1-propanol	2-propanol
1	−1	−1	−1	190	140	230
2	−1	1	−1	2,540	3,410	4,840
3	−1	−1	1	260	235	1,990
4	−1	1	1	3,085	2,560	5,050
5	1	−1	−1	620	340	2,690
6	1	1	−1	2,040	2,580	4,990
7	1	−1	1	2,120	1,620	3,620
8	1	1	1	1,780	1,935	3,600
9	1.682	0	0	140	1,370	2,570
10	−1.682	0	0	210	1,135	360
11	0	1.682	0	1,005	2,210	4,980
12	0	−1.682	0	300	140	120
13	0	0	1.682	4,340	3,590	4,760
14	0	0	−1.682	1,690	2,570	3,090
15	0	0	0	4,390	4,100	5,900
16	0	0	0	5,070	4,220	5,750
17	0	0	0	5,150	4,070	5,880
18	0	0	0	5,050	3,970	5,780
19	0	0	0	4,760	4,170	5,800
20	0	0	0	5,190	4,145	5,765

of action, variables that significantly affect the process are being tested simultaneously in the minimal number of runs. Therefore, this method is less expensive and less time-consuming compared to classical methods. In RSM, geometrical images of a response function are used. These are plots which represent a set of experimental results according to the plan of experiments. The research program was designed in such manner, that it was possible to obtain the necessary information performing the fewest number of analysis possible. Therefore, the research was conducted according to the points of the compositional design, plan which in the form of coded variables is given in Table 1. The number of necessary experiments to conduct was 20 for each of the permeabilization processes, i.e. with the use of ethanol, 1-propanol, 2-propanol.

Results and discussion

Measuring ranges

Table 2 shows measuring ranges for agents affecting the permeabilization process of baker's yeast cells with selected alcohols, determined on the basis of preliminary investigations.

Table 2

Measurement ranges

Measurement ranges	Ethanol			1-Propanol			2-Propanol		
	T [ºC]	S [%]	t [min.]	T [ºC]	S [%]	t [min.]	T [ºC]	S [%]	t [min.]
−1.682	6.6	24.8	7	1.5	3.2	7	6.6	6.4	7
−1	10	35	20	5	10	20	10	20	20
0	15	50	40	10	20	40	15	40	40
1	20	65	60	15	30	60	20	60	60
1.682	23.4	75.2	73	18.5	36.8	73	23.4	73.6	73

Response surface plots

In order to perform an accurate analysis of the process behavior within the limits of the experiment, response surface plots were created. They were based upon experiments conducted in the points of compositional plans (TRAWCZYŃSKA, WÓJCIK 2014, TRAWCZYŃSKA, WÓJCIK 2015). Plots present an effect of two process variables on activity of catalase, assuming that the value of the third variable is constant.

Response surface plot (Fig. 1*a*), depicting catalase activity (i.e., effectiveness of the process) as a function of temperature and alcohol concentration, indicates a gradual increase of activity with the increase of analyzed parameters, up to the alcohol concentration of approx. 50% and the temperature of approx. 15°C. With a further increase of both variables a decrease of enzyme activity can
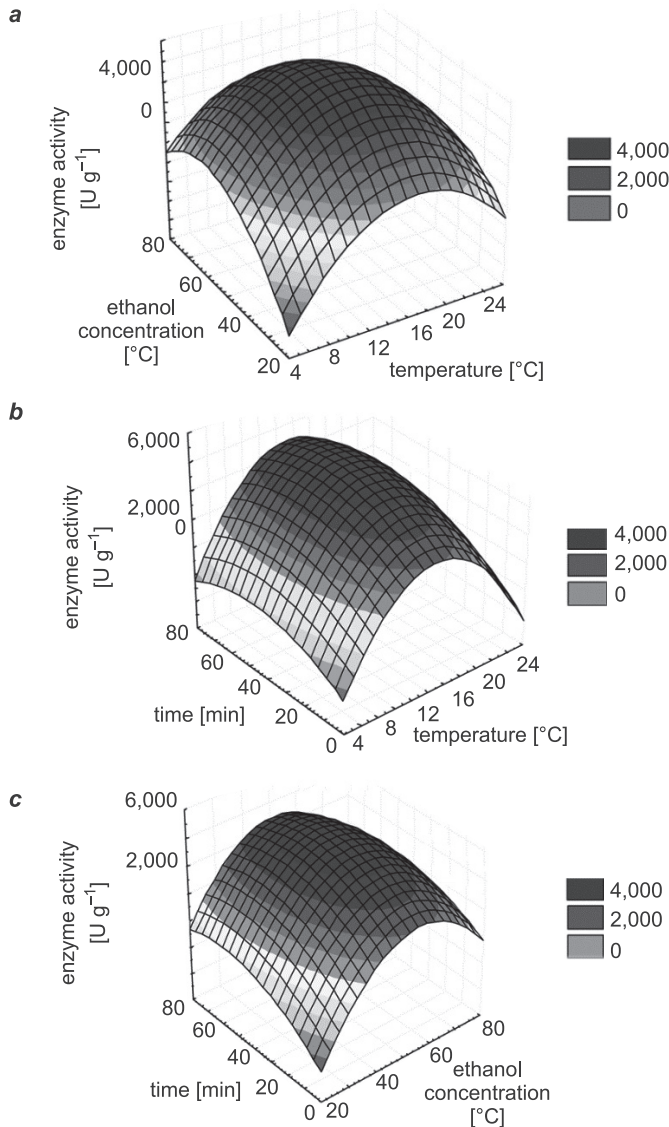

Fig. 1. Response surface plots for permeabilization of baker's yeast cells using etanol; the effect of: *a* – temperature and ethanol concentration, *b* – temperature and permeabilization time, *c* – ethanol concentration and permeabilization time

be noticed. This can be explained by that fact that alcohol concentration and temperature of the process increase up to certain values, for which it is possible to observe the maximal catalase activity. Once the values are exceeded, a correlation can be observed, indicating that the higher the solvent concentration and temperature are, the lower the enzyme activity is. This correlation can be caused by deactivation of the enzyme in higher concentrations of alcohol or higher temperatures. Low effectiveness of permeabilization at low concentrations can be explained by an insufficient amount of solvent.

According to the data presented in Figure 1*b*, the effectiveness of permeabilization of baker's yeast increase with the increase of temperature at which the process has been performed. Once the temperature of approx. 15°C is exceeded the response plot breaks and falls, which can be caused by the thermal deactivation of enzyme. Surfaces in Figure 1*b* and 1*c* depict the influence of the treatment time of the process on its effectiveness. Optimum (4,900–5,100 $U \cdot g^{-1}$) can be observed within a wide range of time. Consequently, this leads to conclusion that the parameter has a slight effect on the process.

The plot presenting the dependence of catalase activity on the concentration of 1-propanol and temperature (Fig. 2*a*) indicates high enzyme activity (4,000 $U \cdot g^{-1}$) within temperature and alcohol concentration ranges of 8–12°C and 15–25% respectively. Below and above those ranges a significant decrease of effectiveness of permeabilization can be noticed. Plot presented in Figure 2*b* is a graphical representation of the dependence of catalase activity on temperature and treatment time of the process at a constant concentration of alcohol solution. Dominant influence of the first of analyzed variables on the shape of the plot is noticeable. Convex response surface possesses a breaking point (2,980–3,100 $U \cdot g^{-1}$) within temperature range of 8–12°C. Figure 2*c* depicting catalase activity in the concentration function of 1-propanol and treatment time of the process is determined mainly by the first parameter. Enzyme activity increases in the concentration ranging from 0 to 15%, and the plot takes the form of a plane with a certain inclination angle. In the areas of higher concentrations a smooth curvature of the plot can be noticed, where the activity maintains the level of 4,000 $U \cdot g^{-1}$. The response plots in Figure 2*b* and 2*c*, similar to the permeabilization with the use of ethanol, present evident optimum within a wide range of time.

Figure 3 depicts 3D response surface plots of catalytic activity depending on temperature, concentration of 2-propanol and treatment time of the process. A parabolic relationship between analyzed parameters of the process is indicated, similar to the permeabilization with the use of ethanol and 1-propanol. According to the data presented in Figure 3a, employing low concentrations of alcohol (i.e. below 30%) at low temperatures does not lead to a significant increase of catalytic activity. Similar to the use of high concentrations of alcohol at high temperatures of the process. High catalase activities (approx. 5,800 $U \cdot g^{-1}$)
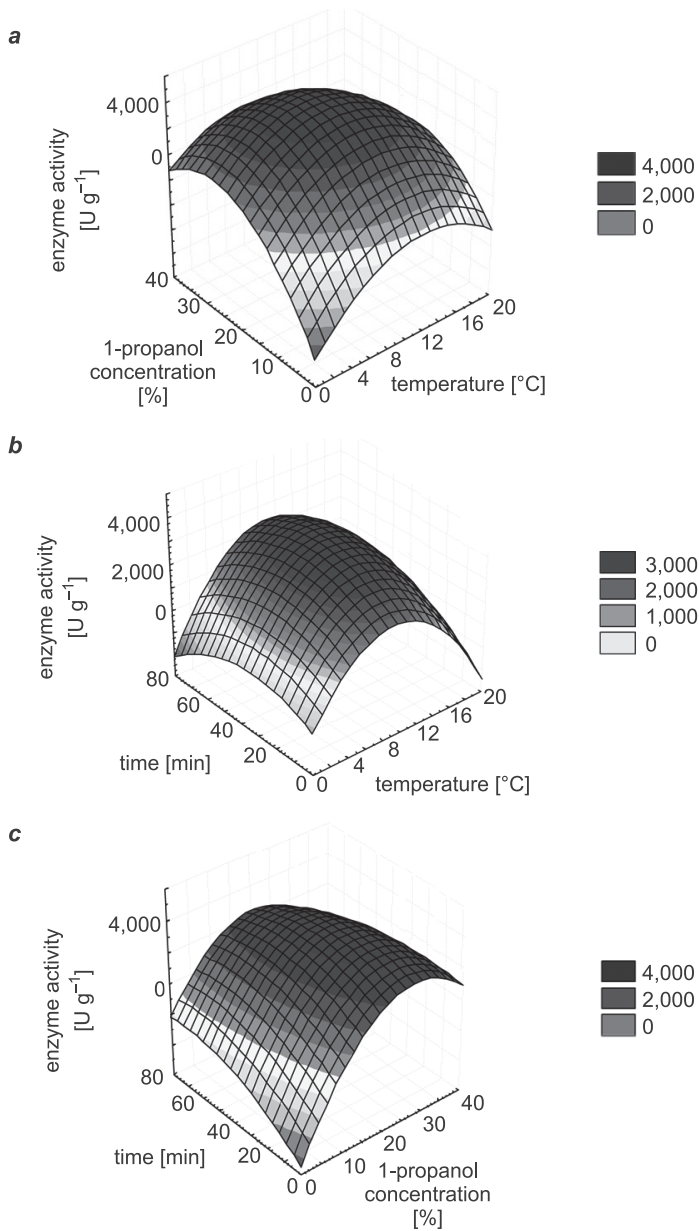
a

b

c

Fig. 2. Response surface plots for permeabilization of baker's yeast cells using 1-propanol; the effect of: *a* – temperature and 1-propanol concentration, *b* – temperature and permeabilization time, *c* – 1-propanol concentration and permeabilization time
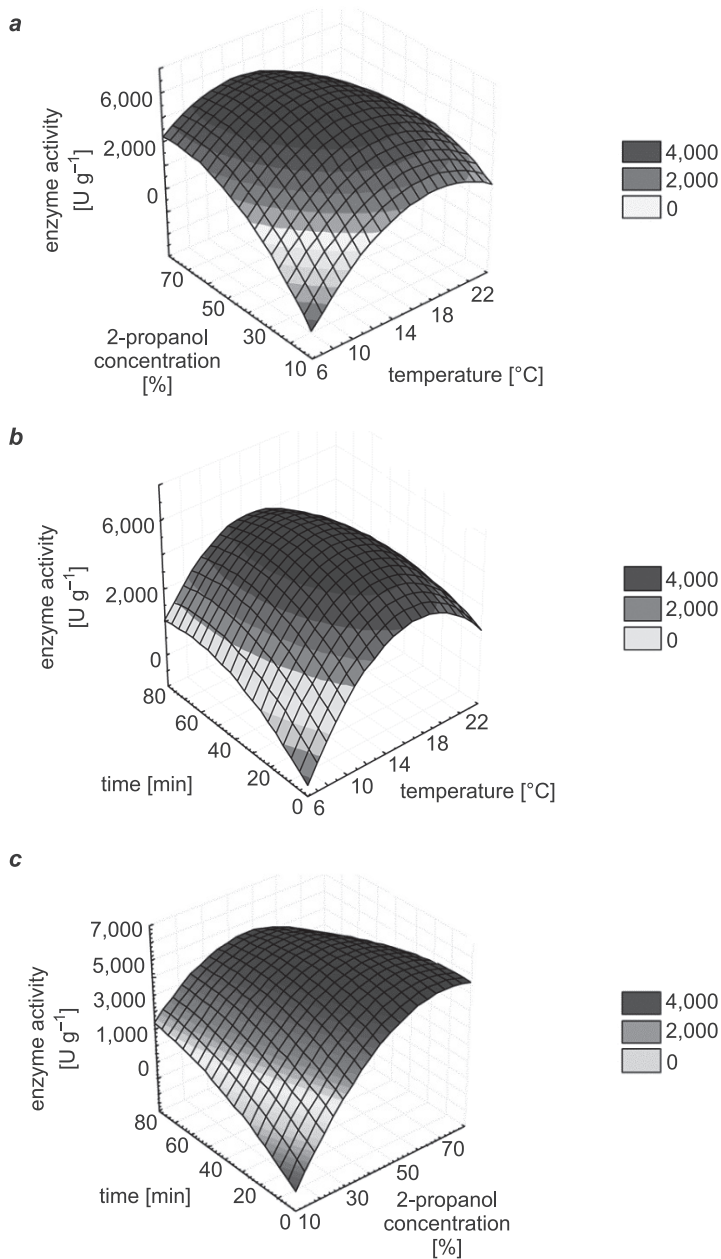
Fig. 3. Response surface plots for permeabilization of baker's yeast cells using 2-propanol;
the effect of: *a* – temperature and 2-propanol concentration, *b* – temperature
and permeabilization time, *c* – 2-propanol concentration and permeabilization time

maintain their level within the temperature range of 10–20°C and 2-propanol concentration range of 40–60%.

Performed tests indicate temperature and alcohol concentration as significant factors affecting the process of permeabilization of baker's yeast. At the same time the tests demonstrate little relevance on the process when it comes to the treatment time. Similar results have been achieved by PANESAR et al. (2007). Those experiments have been conducted with the use of alcohols as a permeabilizing agent, however, in the permeabilization process of yeast cells *Kluyveromyces*, not baker's yeast. Moreover, the effectiveness of the process was based upon measurements of β-galactosidase intracellular activity. By comparing the planes for the permeabilization of baker's yeast with the use of specific alcohols, conclusions can be drawn that 2-propanol enables to achieve the highest enzymatic activity. Nevertheless, yeast cells of similar effectiveness can be achieved employing ethanol in permeabilization process. The advantage of ethanol, however, is the fact that it is safer alcohol, that can be utilized in food industry without any significant restrictions. Thereby, yeast cells permeabilized with ethanol are considered significantly attractive biocatalysts.

Mathematical models

Mathematical models determining dependence of catalase activity (A) on the parameters of permeabilization process form a quadratic equation in three variables (Tab. 3). They can be used in permeabilization process simulation within the tested parameters: temperature (T), concentration of alcohol (S) and time (t).

Table 3

Mathematical models

Ethanol	$A_E = 4{,}919 + 545{\cdot}S_E + 464{\cdot}t_E - 1{,}574{\cdot}T_E^2 - 1{,}405{\cdot}S_E^2 - 570{\cdot}t_E^2 - 512{\cdot}T_E{\cdot}S_E$
1-Propanol	$A_{1P} = 4{,}116 + 851{\cdot}S_{1P} - 1{,}032\,T_{1P}^2 - 1{,}059{\cdot}S_{1P}^2 - 386{\cdot}t_{1P}^2 - 378{\cdot}T_{1P}{\cdot}S_{1P}$ $- 358{\cdot}S_{1P}{\cdot}t_{1P}$
2-Propanol	$A_{2P} = 5{,}784 + 477{\cdot}T_{2P} + 1{,}327{\cdot}S_{2P} - 1{,}343\,T_{2P}^2 - 960{\cdot}S_{2P}^2 - 473{\cdot}t_{2P}^2 -$ $675{\cdot}T_{2P}{\cdot}S_{2P} - 484{\cdot}S_{2P}{\cdot}t_{2P}$

The analysis of variance (ANOVA) results shows that the regressions are highly significant and present good determination coefficients ($R^2_E = 0.974$, $R^2_{1P} = 0.981$, $R^2_{2P} = 0.977$). The closer the R^2 is to 1, the stronger the model is and the better it predicts the response.

Conclusions

Based upon the research, conclusion can be drawn, that alcohol of lower concentration provides better results, along with the increase of temperature in permeabilization process of baker's yeast cells. Applying solvents of too high concentrations contribute to the decrease of permeabilization effectiveness. At high temperatures of approx. 20°C, better results can be achieved by applying lower concentrations of alcohol, along with the increased duration of cells shaking. Treatment time of the process proved to be a parameter bearing little influence on the effectiveness of the process.

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