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# Immobilization of permeabilized cells of baker's yeast for decomposition of $H_2O_2$ by catalase

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Permeabilization is one of the effective tools, used to increase the accessibility of intracellular enzymes. Immobilization is one of the best approaches to reuse the enzyme. Present investigation use both techniques to obtain a biocatalyst with high catalase activity. At the beginning the isopropyl alcohol was used to permeabilize cells of baker's yeast in order to maximize the catalase activity within the treated cells. Afterwards the permeabilized cells were immobilized in calcium alginate beads and this biocatalyst was used for the degradation of hydrogen peroxide to oxygen and water. The optimal sodium alginate concentration and cell mass concentration for immobilization process were determined. The temperature and pH for maximum decomposition of hydrogen peroxide were assigned and are 20°C and 7 respectively. Prepared biocatalyst allowed 3.35-times faster decomposition as compared to alginate beads with non permeabilized cells. The immobilized biocatalyst lost ca. 30% activity after ten cycles of repeated use in batch operations. Each cycles duration was 10 minutes. Permeabilization and subsequent immobilization of the yeast cells allowed them to be transformed into biocatalysts with an enhanced catalase activity, which can be successfully used to decompose hydrogen peroxide.

**Keywords:** cell permeabilization, baker's yeast, hydrogen peroxide, immobilization, biocatalyst.

## INTRODUCTION

For many years now, a large group of scientists have been examining the potential of baker's yeasts as biocatalysts in biotransformations and biosynthesis, because of their abundance and the diversity of intracellular enzymes[1–5]. The use of yeasts as biocatalysts is a promising activity in the food processing, textile, and pharmaceutical industries, as well as in medicine. Yeasts are extremely important in brewing – one of the oldest known food-fermentation processes. In 2017, the global beer production amounted to about 1.95 billion hectoliters, up from 1.3 billion hectoliters in 1998[6]. An increasing production of beer is also connected with producing a large quantity of yeasts. The process which yeasts goes through making beer or wine is identical to that of production of biofuels[7]. They turns sugar into ethanol which can be used as a diesel substitute in vehicles. Moreover, yeasts are the most important phenol biodegraders[8], likewise mixed bacterial and yeast strains are used in diesel oil biodegradation[7]. Plenty of research manuscripts proved that yeasts are very diverse and very important from industrial point of view[9].

Catalase, an enzyme which is abundant in yeast cells[10] and belongs to the group of peroxidases, is able to cause catalytic decomposition of hydrogen peroxide to water and molecular oxygen[11]. $H_2O_2$ is an environmentally friendly oxidizing agent and promising chemical sanitizer for use in the food industry. Unfortunately, its residues have to be decomposed. Usually this is done using an enzyme process employing catalase[9]. Extracted enzymes are sensitive to environmental conditions, and there are difficulties associated with their separation from the reaction medium and reused. In order to avoid this problem cheap and eco-friendly yeast are applied.

The use of whole yeast cells with catalase inside encounters some difficulties due to the low permeability of their cell membranes. The limited diffusion of substrates and products is responsible for the low catalytic activity of the cells. Permeabilization is a way to overcome such difficulties by modifying the cell membrane. According to the available literature, permeabilization is frequently used for transforming cells of microorganisms into biocatalysts with an enhanced activity[12–13]. Under the effect of the permeabilizing agent, structure of the cell membrane is modified so that forming pores enable an unobstructed exchange of molecules such as substrate or product of catalyzed reaction. Among many known techniques, permeabilization with use of chemical agents is most popular in the art[14–16]. Previous research works on the subject of permeabilization have proved that highest baker's yeast cells catalase activity was achieved using isopropyl alcohol[17].

Earlier studies on a biocatalyst in the form of permeabilized yeast cells in which the activity of intracellular catalase is enhanced gave extremely promising findings. However, it is hard to use such cells repeatedly in a larger scale because the processes of their separation from the reaction medium are very expensive and time-consuming. Conventional techniques for separation of microorganisms in a fast and inexpensive way include immobilization by means of carriers. Calcium alginate gel entrapment is very popular since gelation can be easily performed under mild conditions. In whole cell immobilization, high cell loading is required to obtain high activity of biocatalyst. This study aims to determine the conditions for producing alginate beads with permeabilized yeast cells inside, in which catalase activity will have the highest value.

## EXPERIMENTAL

### Materials and methods

One and the same batch of compressed baker's yeast (Saccharomyces cerevisae), obtained locally, was stored in the refrigerator at temperature below 5°C and used

within 1 week. Isopropyl alcohol, hydrogen peroxide and other chemicals were purchased from POCH S.A. (Polish Chemicals Reagents). Sodium alginate was obtained from Sigma-Aldrich.

**Permeabilization of yeast cells**

The permeabilization was carried out in accordance with the method determined in previous studies under optimal conditions[18]. The process was conducted under optimal conditions using isopropyl alcohol as a permeabilizing agent. The solution (53.7% v/v) was prepared by mixing alcohol with phosphate buffer (pH 7). One gram (wet wt.) of yeast cells was suspended in 20 g of alcohol. The contents were mixed in water bath at constant temperature 15.6°C for 40 min. After that cells were collected by centrifugation and washed with phosphate buffer (pH 7,5 mL per wash). The treated cells were used for all future experiments.

**Immobilization of the permeabilized cells**

Both permeabilized and non-permeabilized yeast cells were immobilized by entrapment. In typical immobilization experiment the yeast was mixed with a sodium alginate solution in such a way that the final concentration of yeast cells was 50 g/L and final concentration of sodium alginate was 2%. Calcium chloride aqueous solution (1%) was used as a cross-linking agent. The suspensions of yeast cells, suspended in solutions of sodium alginate was added dropwise to 200 mL $CaCl_2$ using a peristaltic pump. The beads were allowed to harden for 30 min and then were separated, washed by distilled water to remove excess ions and yeast cells. Next the beads were wiped with filter paper, examined under a microscope (average diameter 2 mm) and stored in a 0,1% $CaCl_2$ solution.

**Conditions of immobilization process**

At the beginning, research was focused on looking for conditions of immobilization process, which enable preparation of biocatalysts with high enzymatic activity. Two most important parameters were analyzed: cell mass concentration and sodium alginate concentration. The published research findings shows that 2% sodium alginate solution is commonly used in immobilization. Therefore, first biocatalysts were made in such a way that the final concentration of sodium alginate was fixed (2%) but final cell mass concentration was in the range of 10–150 g/L. These alginate beads were used in catalase activity assays during decomposition of hydrogen peroxide under a specified set of conditions.

Next it was checked whether using an alginate solution with a different levels of concentration would give biocatalyst with higher catalase activity. The experiments were conducted at various sodium alginate concentrations in the range of 1,5–3,5% (w/v). A solution of polysaccharides was made by mixing sodium alginate powder with water and stirring continuously for about 5 h. The solution was stored in the refrigerator in order to inhibit microbial growth. The solution with fixed concentration of cells were used to formulate alginate beads. Afterwards biocatalysts were used for decomposition of $H_2O_2$ and then catalase activity was calculated.

**Activity of permeabilized immobilized yeast cells**

Catalase activity of immobilized biocatalyst was assayed similar to the method by Beers and Sizer[19]. Spectrophotometer Helios was used to monitor the decomposition of $H_2O_2$, through the measurement of the decrease in absorbance at 240 nm. The initial concentration of hydrogen peroxide was chosen to be 15 mM, to ensure a good spectrophotometric monitoring of substrate concentration. One gram of biocatalyst was immersed in 50 mL of buffered hydrogen peroxide (pH 7) and the decomposition reaction was carried out at 20°C until at least one-half of substrate was destroyed. The substrate concentrations were calculated using the Lambert–Beer law, with extinction coefficient 39,4 $M^{-1}cm^{-1}$ [20]. Activity of permeabilized immobilized yeast cells was determined as the average rate of decomposition of hydrogen peroxide. Empty alginate beads were used as a control, they do not cause any decomposition of hydrogen peroxide.

**Decomposition of hydrogen peroxide by biocatalyst**

The effect of pH on the decomposition of hydrogen peroxide by biocatalyst was assessed over a range of 4–9. In all experiments the phosphate buffer was used. The other conditions were fixed in order to decide in which pH the activity of yeast catalase will be the highest. Subsequent experiments were carried out at different temperature values (10–40°C) in buffered systems at the above determined pH. The results were used to fix a temperature value for all future experiments.

**Hydrogen peroxide degradation in batch process**

In order to comparison two type of biocatalysts the degradation of $H_2O_2$ in a batch mode was carried out. The first was in the form of alginate beads with permeabilized yeast cells inside, the second with nonpermeabilized. The biocatalysts were introduced into a 50 mL 15 mM hydrogen peroxide solution in bioreactor and gently mixed in a laboratory shaker. The bioreactor was operated at constant conditions – temperature 20°C and pH 7. The decomposition of $H_2O_2$ was followed directly by the decrease in absorbance at 240 nm in a spectrophotometer. The time required for the concentration of hydrogen peroxide to decrease to one-half was measured. After reaching a suitable conversion of $H_2O_2$, the biocatalysts were separated from the reaction medium. The average rate of decomposition of hydrogen peroxide was determined based on measurements of three consecutive uses of biocatalyst to determine the effectiveness of immobilized permeabilized yeast cells.

**Reusability of the permeabilized immobilized cells**

To assess the possibilities of repeated use of immobilized cells, studies were performed as described below. Thirty beads containing entrapped cells were used repeatedly for 15 mM hydrogen peroxide degradation in 25 mL solution. The contents were mixed and incubated (20°C, pH 7) for 10 min under shaking conditions. Then solution was decanted off and analyzed for residual hydrogen peroxide by the spectrophotometric method. A sieve was used to separate beads. The biocatalyst was rinsed with distilled water. After that water on the surface of beads was removed using filter paper. Then

they were added to fresh hydrogen peroxide solution for a new process. This action was repeated 27-times.

## RESULT AND DISCUSSION

With the permeabilization of yeast cells by isopropyl alcohol it is possible to achieve intracellular activity of catalase 62-times higher than with not treated yeast cells[14]. Permeabilization process was not complicated. It required simple equipment and was able to be carried out under mild condition (15.6°C, pH 7) within a short period of 40 min, using readily available and inexpensive alcohol. Furthermore, isopropyl alcohol permeabilized cells retained catalase activity for a quite long period. Therefore, these permeabilized cells could be used as a source of catalase for different applications. In order to increase possibility of using them in industrial conditions, immobilization process was used. Immobilization of yeast in solid structures creates a material of better mechanical properties such as strength, rigidity and porosity necessary for its use in flow and batch procedures[21, 22]. Mild conditions of the process and simplicity of methodology makes immobilization with alginate as a carrier the most common[23]. Therefore, this study used method of entrapping in alginate gel to obtain immobilized biocatalyst, able to decompose hydrogen peroxide with high efficiency.

### Effect of cell mass concentration on immobilization of the permeabilized cells

Cell mass concentration of beads affects efficiency of the immobilized system. Yeast cells were mixed with a sodium alginate solution in such a way that the final concentration of cells was within range of 10–150 g/L and final concentration of sodium alginate was 2%. Figure 1A shows direct dependency of catalase activity on increase of cell mass concentration up to 50 g/L which is the maximum value. As shown in Fig. 1A the use of cell with concentration higher than 50 g/L does not affect catalytic performance of beads. Further, too high value of cell mass concentration adversely affects mechanical strength and diffusional characteristics of the beads[24].

### Effect of sodium alginate concentration on immobilization of the permeabilized cells

Alginate concentration is a major parameter for general entrapment[25]. The effect of sodium alginate concentration on the catalase activity in immobilized permeabilized yeast cell was determined, and the results were presented in Fig. 1B. When alginate concentration was increased from 1.5 to 3.5% the highest catalase activity was found to be 2% (w/v). Sodium alginate in the concentration range 2–3% has been used for immobilization of various cells like micro-alga Prototheca zopfii[26], bacteria Methylosinus trichosporium $OB_3b$[27], Teredinobacter turnirae[28] and also yeast Candida guilliermondii[29]and Saccharomyces cerevisiae cells[30].

### Effect of pH and temperature

Temperature and pH are the environmental factors that affect enzyme reactions. The optimal value of these parameters of reaction coincides with the point where the catalase activity is at the maximum value. Moreover

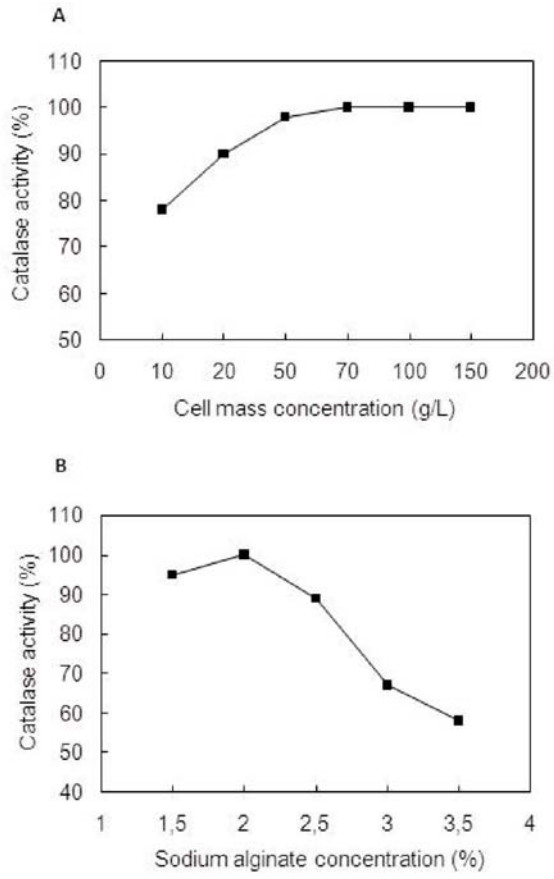


**Figure 1.** Effect of A) cell mass concentration and B) sodium alginate concentration on the decomposition of $H_2O_2$ by catalase

,the removal of excess hydrogen peroxide is most often carried out in mixtures of various components with different pH. That's why knowledge of changes of biocatalyst activity at different pH and temperature is so important. For these reasons, the effect of pH and temperature on the immobilized permeabilized cells was analyzed.

Alginate beads were prepared in such a way that final concentration of sodium alginate was 2% and final concentration of permeabilized yeast cells was 50 g/L, as set out above. One gram biocatalyst was used in decomposition of hydrogen peroxide (50 mL, 15 mM, 20°C) at different pH. The catalase activity was determined of. The results obtained are depicted in the graph further shown by Fig. 2A. Biocatalyst exhibits high activity of catalase in the pH range of (6–8) with maximum, at pH of 7. The same results were obtained when catalase from intact cells of Saccharomyces cerevisiae was used[31, 32].

In this work the effect of temperature on the activity of alginate beads with permeabilized yeast cells inside was studied by carrying out the reaction of decomposition of $H_2O_2$ (pH 7) at various temperature. Results are given in Fig. 2B. The optimum temperature range of 20–25°C is consistent with the results obtained by other authors[33]. Therefore, all future experiments use temperature of 20°C. Cells permeabilized with toluene and then immobilized in sodium alginate show a similar dependence of catalase activity on temperature. However, according to earlier reports, 2-propanol is much more effective permeabilizing agent and allows to increase the activity of catalase in yeast cells 60-fold higher than toluene[18].

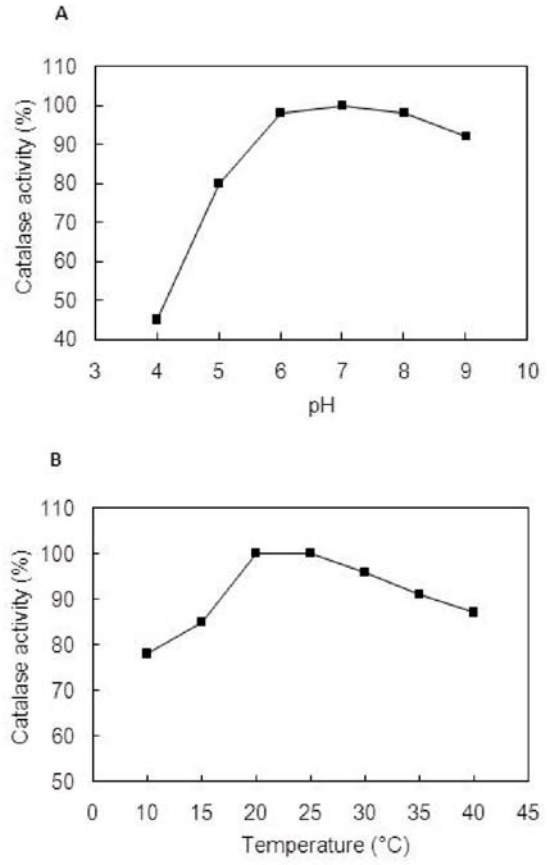


**Figure 2.** Effect of A) pH and B) temperature on the decomposition of $H_2O_2$ by catalase

### Degradation of hydrogen peroxide by immobilized cells

The hydrogen peroxide decomposition average rates for permeabilized and non-permeabilized immobilized yeast cells were compared, and the results are presented in Table 1.

The use of immobilized cells after permeabilization allowed 3.35-times faster decomposition of hydrogen peroxide into water and oxygen as compared to immobilized cells before permeabilization. Degradation of $H_2O_2$ takes place in the interior of cells which are inside of the alginate beads, Therefore, substrates and products diffuse through the pores of the cell walls and a carrier. The observed rate of reaction will differ from the intrinsic rate of reaction because of resistance to this pore diffusion. The effectiveness factor as a ratio of the reaction rate of permeabilized immobilized cells for the reaction rate of free cells was 0.1.

**Table 1.** The hydrogen peroxide decomposition average rates for non-permeabilized and permeabilized immobilized yeast cells

| Hydrogen peroxide decomposition average rates [mol/dm$^3$ * min] * $10^{-4}$ | |
|---|---|
| Permeabilized cells | Non-permeabilized cells |
| 2.14 | 0.64 |

### Reusability of the permeabilized immobilized cells

To determine possibility of repeated use, alginate beads with entrapped cells after permeabilization were tested (Fig. 3). Activity of freshly prepared beads in the third run was defined as 100%. Data in Figure 3 indicate that

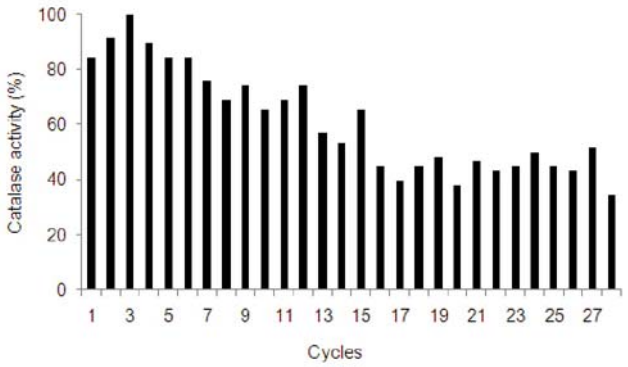


**Figure 3.** Degradation of $H_2O_2$ using alginate beads containing entrapped permeabilized baker's yeast cells in a batch process

immobilized permeabilized cells lost ca. 30% activity after 10 repeated operations. After 20 reaction cycles deterioration of the structure was observed and thereby caused inconsistent and irreproducible experimental results. Nevertheless, the results are satisfactory considering the difficulties associated with the use of enzyme extracts, and in particular the inability to use them again.

## CONCLUSION

To increase usefulness of permeabilized yeast cells in industry, attempts were made to determine the efficiency of immobilization of cells in a sodium alginate. Insufficient knowledge of the immobilization parameters and methods for their control lead to unsubstantiated elimination of promising immobilization techniques for industrial use. For this reason the optimal conditions for producing alginate beads with permeabilized yeast cells inside was determined. The optimal sodium alginate concentration and cell mas concentration are respectively 2% (w/v) and 50 g/L. For decomposition of hydrogen peroxide using biocatalyst beads it is recommended to conduct the process at temperature of 20°C and pH 7.

Entrapped cells after permeabilization allow easier and faster separation of reaction environment as well as to achieve 3.35-fold quicker decomposition of hydrogen peroxide in comparison with immobilized non-permeabilized cells. Immobilized permeabilized cells lost ca. 30% activity after 10 repeated uses. Therefore, such biocatalyst can find applications in the industry in the removal of excess $H_2O_2$.

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## LITERATURE CITED

1. Pscheidt, B. & Glieder, A. (2008). Yeast cell factories for fine chemical and API production. *Microb. Cell Fact*. 7(1), 25. DOI: 10.1186/1475-2859-7-25.

2. Pratap, U.R., Jawale, D.V., Londhe, B.S. & Mane, R.A. (2011). Baker's yeast catalyzed synthesis of 1,4- benzothiazines, performed under ultrasonication. *J. Mol. Catal. B- Enzym.* 68(1), 94–97. DOI: 10.1016/j.molcatb.2010.09.018.

3. Hounga, J.Y. & Liau, J.S. (2006). Mathematical modeling of asymmetric reduction of ethyl 4-chloro acetoacetate

by bakers' yeast. *Enzyme Microb. Tech*. 38(7), 879–886. DOI: 10.1016/j.enzmictec.2005.02.028.

4. Fow, K.L., Poon, L.C.H., Sim, S.T., Chuah, G.K. & Jaenicke, S. (2008). Enhanced asymmetric reduction of ethyl 3-oxobutyrate by baker's yeast via substrate feeding and enzyme inhibition. *Eng. Life Sci.* 8(4), 372–380. DOI: 10.1002/elsc.200700052.

5. Yu, M.A., Hou, Y., Gong, G.H., Zhao, Q., Zhu, X.B., Jiang, L., Yang, X.L. & Liao, F. (2009). Effects of industrial storage on the bioreduction capa city of brewer's yeast. *J. Ind. Microbiol. Biot*. 36(1), 157–162. DOI: 10.1007/s10295-008-0483-x.

6. FAO. (2018). World food and agriculture – statistical pocketbook 2018. Rome. 254 pp. Licence: CC BY-NC-SA 3.0 IGO.

7. Miranda, R.C., Souza, C.S., Gomes, E.B., Lovaglio, R.B., Lopes, C.E. & Sousa, M.F. (2007). Biodegradation of diesel oil by yeasts isolated from the vicinity of Suape Port in the State of Pernambuco – Brazil. Braz. *Arch. Biol. Technol.* 50(1), 147–152. DOI: 10.1590/S1516-89132007000100018.

8. Karimi, M., Hassanshahian, M., Karimi, M. & Hassanshahian, M. (2016). Isolation and characterization of phenol degrading yeasts from wastewater in the coking plant of Zarand, Kerman. *Braz. J. Microbiol*. 47(1), 18–24. DOI: 10.1016/j.bjm.2015.11.032.

9. Kaushal, J., Mehandia, S., Singh, G., Raina, A. & Arya, S.K. (2018). Catalase enzyme: application in bioremediation and food industry. Biocatal. Agric. Biotechnol. 16, 192–199. DOI: 10.1016/j.bcab.2018.07.035.

10. Venkateshwaran, G., Somashekar, D., Prakash, M.H., Agrawal, R., Basappa, S.C. & Joseph R. (1999). Production and utilization of catalase using Saccharomyces cerevisiae. *Process Biochem*. 34(2), 187–191. DOI: 10.1016/S0032-9592(98)00087-9.

11. Raducan, A., Cantemir, A.R., Puiu, M. & Oancea, D. (2012). Kinetics of hydrogen peroxide decomposition by catalase: hydroxylic solvent effects. *Bioproc. Biosyst. Eng*. 35(9), 1523–1530. DOI: 10.1007/s00449-012-0742-0.

12. Presecki, A.V. & Vasić–Racki, D. (2005). Production of L–malic acid by permeabilized cells of commercial Saccharomyces sp. Strains. Biotechnol. Lett. 27(23–24), 1835–1839. DOI: 10.1007/s10529-005-3890-3.

13. Yu, M.A., Wei, Y.M., Zhao, L., Jiang, L., Zhu, X.B. & Qi,W. (2007). Bioconversion of ethyl 4-chloro-3-oxobutanoate by permeabilized fresh brewer's yeast cells in the presence of allyl bromide. *J. Ind. Microbiol. Biot*. 34(2), 151–156. DOI: 10.1007/s10295-006-0179-z.

14. Panesar, P.S., Panesar, R., Singh, R.S. & Bera, M.B. (2007). Permeabilization of yeast yells with organic solvents for β–galactosidase activity. *Res. J. Microbiol*. 2(1), 34–41. DOI: 10.3923/jm.2007.34.41.

15. Abraham, J. & Bhat, S.G. (2009). Permeabilization of baker's yeast with N–lauroyl sarcosine. *J. Ind. Microbiol. Biotechnol*. 35(8), 799–804. DOI: 10.1007/s10295-008-0350-9.

16. Sekhar, S., Bhat, N. & Bhat, S.G. (1999). Preparation of detergent permeabilized Bakers' yeast whole cell catalase. Proc. Biochem. 34(4), 349–354. DOI: 10.1016/S0032-9592(98)00105-8.

17. Trawczynska, I. & Wojcik, M. (2014). Application of Response Surface Methodology for optimization of permeabilization process of baker's yeast, *Pol. J. Chem. Technol*. 16(2), 31–35. DOI: 10.2478/pjct-2014-0026.

18. Trawczynska, I. (2015). Research and modeling of the yeast cells permeabilization process using selected alcohols. Published doctoral dissertation. West Pomeranian University of Technology Szczecin.

19. Beers, R.F. & Sizer, I.W. (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem*. 195(1), 133–140.

20. Chance, B. (1950). The reactions of catalase in the presence of the notatin system. *Biochem. J*. 46(4), 387–402.

21. Idris, A. & Suzana, W. (2006). Effect of sodium alginate concentration, bead diameter, initial pH and temperature on lactic acid production from pineapple waste using immobilized Lactobacillus delbrueckii. *Process Biochem*. 41(4), 1117–1123. DOI: 10.1016/j.procbio.2005.12.002.

22. Liouni, M., Drichoutis, P. & Nerantzis, E.T. (2007). Studies of the mechanical properties and the fermentation behavior of double layer alginate–chitosan beads, using Saccharomyces cerevisiae entrapped cells. *World J. Microb. Biot*. 24(2), 281–288. DOI: 10.1007/s11274-007-9467-7.

23. Gokgoz, M. & Yigitoglu, M. (2011). Immobilization of Saccharomyces Cerevisiae on to modified carboxymethylcellulose for production of ethanol. *Bioproc. Biosyst. Eng*. 34(7), 849–857. DOI: 10.1007/s00449-011-0535-x.

24. Suenaga, T., Aoyagi, R., Sakamoto, N., Riya, S., Ohashi H., Hosomi M., Tokuyama, H. & Terada, A. (2018). Immobilization of Azospira sp. strain I13 by gel entrapment for mitigation of $N_2O$ from biological wastewater treatment plants: Biokinetic characterization and modelling. *J. Biosci. Bioeng*. 126(2), 213–219. DOI: 10.1016/j.jbiosc.2018.02.014.

25. Lee, K.H., Choi, I.S., Kim, Y.G., Yang, D.J. & Bae, H.J. (2011). Enhanced production of bioethanol and ultrastructural characteristics of reused Saccharomyces cerevisiae immobilized calcium alginate beads. *Bioresource Technol*. 102(17), 8191–8198. DOI: 10.1016/j.biortech.2011.06.063.

26. Suzuki, T., Yamaguchi, T. & Ishida, M. (1998). Immobilization of Prototheca zopfii in calcium alginate beads for the degradation of hydrocarbons. *Process Biochem*. 33(5), 541–546. DOI: 10.1016/S0032-9592(98)00022-3.

27. Taylor, A., Molzahn, P., Bushnell, T., Bushnell, T., Cheney, C., LaJeunesse, M., Azizian, M. & Semprini, L. (2018). Immobilization of Methylosinus trichosporium $OB_3b$ for methanol production. *J. Ind. Microbiol. Biotechnol*. 45(3), 201–211. DOI: 10.1007/s10295-018-2010-z.

28. Elibol, M. & Moreira A.R. (2003). Production of extracellular alkaline protease by immobilization of the marine bacterium Teredinibacter turnirae. *Process Biochem*. 38(10), 1445–50. DOI: 10.1016/S0032-9592(03)00024-4.

29. Carvalho, W., Silva, S.S., Converti, A., Vitolo, M., Felipe, M.G.A., Roberto, I.C., Silva, M.B. & Manchilha, I.M. (2002). Used of immobilized Candida yeast cells for xylitol production from sugarcane bagasse hydrolysate. *Appl. Biochem. Biotech*. 98(1–9), 489–496. DOI: 10.1385/ABAB:98-100:1-9:489.

30. Duarte, J.C., Rodrigues, J.A., Moran, P.J., Valença, G.P. & Nunhez, J. R. (2013). Effect of immobilized cells in calcium alginate beads in alcoholic fermentation. AMB Express. 3, 31. DOI: 10.1186/2191-0855-3-31.

31. Kaushal, J., Seema, Singh, G. & Arya, S.K. (2018). Immobilization of catalase onto chitosan and chitosan-bentonite complex: A comparative study. Biotechnol. Rep. 18, 251–258. DOI: 10.1016/j.btre.2018.e00258.

32. Seah, T.C.M. & Kaplan, J.G. (1973). Purification and properties of the catalase of bakers' yeast. *J. Biol. Chem*. 248(8), 2889–2893.

33. D'Souza, S.F., Deshpande, A. & Nadkarni, G.B. (1987). Effect of permeabilization on the thermostability of catalase in immobilized yeast cells. *Biotechnol. Lett*. 9(9), 625–628. DOI: 10.1007/BF01033199.