sciendo

Polish Journal of Chemical Technology, 20, 4, 44—48, 10.2478/pjct-2018-0053

Membrane reactor for enzymatic depolymerization – a case study based on protein hydrolysis

Andrzej Noworyta, Anna Trusek*, Maciej Wajsprych

Wroclaw University of Science and Technology, Division of Bioprocess and Biomedical Engineering, Wybrzeze Wyspianskiego 27, 50-373 Wrocław, Poland
**Corresponding author: e-mail: anna.trusek@pwr.edu.pl*

The efficiency of enzymatic depolymerization in a membrane reactor was investigated. The model analysis was performed on bovine serum albumin hydrolysis reaction led by three different enzymes, for which kinetic equations have different forms. Comparing to a classic reactor, the reaction yield turns out to be distinctly higher for all types of kinetics. The effect arises from increasing (thanks to the proper selectivity of the applied membrane) the concentration of reagents in the reaction volume. The investigations indicated the importance of membrane selectivity election, residence time and at non-competitive inhibition the substrate (biopolymer) concentration in feed stream. Presented analysis is helpful in these parameters choice for enzymatic hydrolysis of different biopolymers.

Keywords: enzymatic membrane reactor, membrane selectivity, volume enzyme immobilization, biopolymer hydrolysis, process efficiency.

INTRODUCTION

Reactions of an enzymatic depolymerization are specific series-parallel reactions. Each polymer consists of oligomers, which are substrates for further depolymerization[1, 2]. Reaction system for hundreds of mers in molecule of biopolymers like proteins, cellulose, lignin etc. is very complex. A mer is the smallest unit e.g. an amino acid inside a peptide chain. In the case when biopolymer is composed of N - mers, the reaction system has N-1 substrates. Within different mers, the quantity of reagents increases, reaching even up to thousands. This description shows a complexity and the scale of problem.

In order to run enzymatic reaction effectively, immobilization of biocatalyst can be performed. Among various methods of enzyme immobilization, a special kind is a volume immobilization in membrane bioreactor[3–5]. Due to the size of enzyme molecules, an ultra/nanofiltration membrane is able to retain them in the reaction volume, without special techniques and reagents causing enzyme inactivation[6–7].

As it was mentioned, in a reaction of depolymerization many reagents with wide range of molecular mass and size are involved at once[8]. It's a symptom for the application a membrane with allowing to retain in the reaction volume not only biocatalyst molecules, but also partially hydrolyzed substrate i.e. oligomers with relativity long bonds. It causes an increase in their concentration and in many cases simultaneously an increase in the reaction rate. Only highly hydrolyzed oligomers with short and medium long chains, which are usually expected product of depolymerization should be present in the outflow stream[9]. Additionally, depolymerization reactions take place very often with an inhibition by low molecular weight products[10]. Molecules of those sizes are not retained by an ultra/nanofiltration membrane, hence can be taken out of the reaction volume and consequently the decrease in their concentration should increase the reaction rate.

Model of enzymatic depolymerization reaction

The model of an enzymatic depolymerization reaction was developed based on chemical bonds mer-mer hydrolysis[11]. The model properly describes the mechanism of the reaction since chemical bonds take part in the reaction; not chemical compounds as it is commonly said so. In the case of most biopolymers (including proteins), bonds are not structurally equal (i.e. not all bonds are equally susceptible to hydrolysis by a given enzyme). Therefore, number of bonds of a given sequence of mers and their location in the chain determine the kinetics. For a given length of chain, dozens sequences of mers at different levels of the reaction susceptibility to hydrolysis may occur.

Description of such a complex reaction system needs a few assumptions:

1. The sequence of mers in the hydrolyzed biopolymer and the substrate affinity of the applied enzyme are known;
2. As a result of a single contact of the substrate molecule with the enzyme molecule, one bond is cleaved;
3. Each of the chemical bonds depending on the susceptibility to hydrolysis by the given enzyme has a defined reactivity coefficient (β);
4. Hydrolysis of all bonds is described by the same form of kinetic equation including reactivity coefficients;
5. Each mer of a given biopolymer has the same (average) molar mass.

A detailed description of the model with the algorithm of calculations is presented in the previous publication[11]. The model is based on the general kinetic equation taking into account concentrations of all reagents, reactivity of the individual bonds, inhibition by any component of the reaction mixture and the enzyme inactivation over the time. The model allows to determine the concentration of all components (substrate, by-products, final products) and the general degree of biopolymer hydrolysis. There is a very large number of reagents in the system; e.g. for a polymer that contains 50 reactive bonds the number of reagents is 1275.

Process in membrane reactor

Scheme of membrane bioreactor in continuous flow mode is shown in Fig. 1.

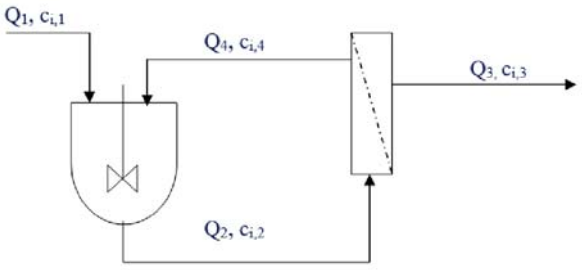

Figure 1. Scheme of membrane reactor

At steady state the balance of reagent *i* presents the equation

$$Q_1 \cdot c_{i,1} = Q_3 \cdot c_{i,2} \cdot (1 - R_i) + V_R \cdot r_i(c_2)$$

When $Q_1 = Q_3$, a relationship is obtained

$$\tau = \frac{V_R}{Q_1} = \frac{c_{i,1} - (1 - R_i)c_{i,2}}{r_i(c_2)} \quad (2)$$

where[12]

$$R_i = 1 - \frac{c_{i,3}}{c_{i,2}} \quad (3)$$

The aim of this study was to analyze the performance of a membrane reactor based on the kinetic data obtained for reactions carried out in a stirred tank reactor and the previously presented model[11]. Three different enzymes showing different protein hydrolysis kinetics were analyzed. Albumin was used as the model protein.

EXPERIMENTAL

Material and methods

Reaction of hydrolysis of bovine serum albumin (Sigma-Aldrich, CAS Number: 9048-46-8) as a typical protein (584 amino acids of a known sequence in the chain) was examined with three different enzymes. The reactions were carried out in a thermostated stirred (250 rpm) bioreactors. Subtilisin (P4860, Sigma), thermolysin (Cat#58656, Calbiochem) and pepsin (P7000 Sigma) were tested.

The solution of the substrate (at fixed concentration in range 2.5–20 g/L) was preincubated with the aim of the protein unfolding. For this purpose bovine serum albumin (BSA) dissolved in the buffer was placed into the reactor at temperature of 60°C for 30 min. After that, the temperature was decreased to the reaction temperature and the enzyme solution in given buffer was added. During this process samples were withdrawn from the reactor in certain time intervals, cooled rapidly in ice water bath and then kept at –20°C for further analyses. The quantity and quality of obtained peptide products were determined using SE-HPLC.

Molecular weight distribution of albumin hydrolyzates was analyzed by size exclusion high performance liquid chromatography under isocratic conditions using a 300 x 7.8 mm BioSep-SEC- s2000 column combined in series with Yarra-3u-SEC-2000 (Phenomenex, USA) on an HPLC system (Shimadzu, USA). Samples, filtered through 0.22 μm syringe filter, were eluted with 0.1 M phosphate buffer pH 6.8 at 25°C for 60 min. The flow rate was 0.6 mL/Min. and peak absorbance was monitored at 214 nm. Bovine serum albumin (66.0 kDa), ovalbumin (44.3 kDa), carbonic anhydrase (29.0 kDa), α-lactalbumin (14.2 kDa), cytochrome C (12.4 kDa), aprotinin (6.5 kDa) and vitamin B12 (1.3 kDa) were used for standard curve determination: (Log (Mw)=-0.1296.t+5.2143). The percentage quantity (area under peak) of the determined molecular weight fractions was derived from the HPLC software (LabSolutions LC/GC version 5.51, Shimadzu Corporation, Kyoto, Japan).

For the tested systems, reactive bonds in the bovine serum albumin chain were estimated using the BIOPEP base. The kinetic equation and its constants were experimentally determined for each enzyme using the computer program and the method described previously[11]. The reaction yield was estimated in order to compare expected and obtained degree of hydrolysis.

Kinetics of bovine serum albumin hydrolysis by subtilisin

It has been experimentally demonstrated that this is 1st order reaction without inhibition[13], which kinetics (at 50°C, pH 7,0, enzyme concentration 0.01 g/L) is described by:

$$\frac{dc_i}{dt} = k \cdot \left(\sum_{j=i+1}^{N} c_j \cdot (\beta_{j,i} + \beta_{j,j-1}) - c_i \cdot \sum_{m=1}^{i-1} \beta_{i,m} \right) \quad (4)$$

For this enzyme the chain of bovine serum albumin consists of 49 reactive bonds (causing of being 1125 reagents), which may be divided into the three groups. As a result of the experimental data regression analysis (Fig. 2), values of the reactivity coefficient were obtained as follows: 6 bonds with β = 1.0 (very reactive bonds), 17 bonds with β = 0.031, 26 bonds with β = 0.0049 (very slow reaction of their hydrolysis) and the reaction rate constant k = 1.78 [1/h].

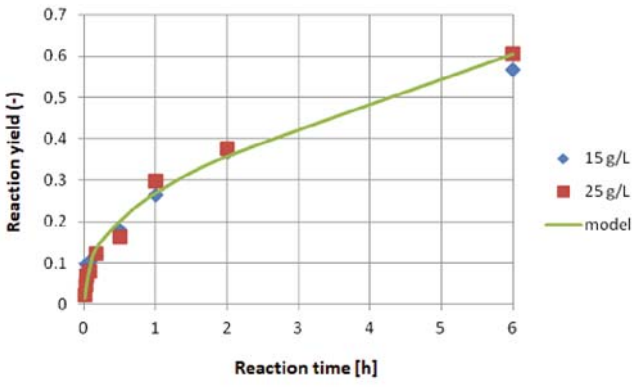

Figure 2. Dependence of the reaction yield on the reaction time for the albumin-subtilisin system (50°C, pH 7.0, enzyme concentration 0.01 g/L, bovine serum albumin 15 and 25 g/L)

Kinetics of bovine serum albumin hydrolysis by thermolysin

It has been experimentally proven that it is reaction of 1st order with non-competitive inhibition. As an inhibitor, a short-chain peptide fraction (m<5) was assumed[14].

Kinetics (at 60°C, pH 7.0, enzyme concentration 0.005 g/L) is described by:

$$\frac{dc_i}{dt} = k \cdot \left(1 - \frac{c_{inh}}{K_{inh}}\right)\left(\sum_{j=i+1}^{N} c_j \cdot (\beta_{j,i} + \beta_{j,j-1}) - c_i \cdot \sum_{m=1}^{i-1} \beta_{i,m}\right) \quad (5)$$

For this enzyme the chain of the bovine serum albumin consists of 49 reactive bonds (resulting in the presence of 1125 reagents), which may be divided into the two groups. As a result of the experimental data regression analysis (Fig. 3), values of the reactivity coefficient were obtained as follows: 5 bonds with β = 1, 44 bonds with β = 0.17, reaction rate constant k = 14.4 [1/h]) and inhibition constant K_{inh} = 0.0145 [g/L].

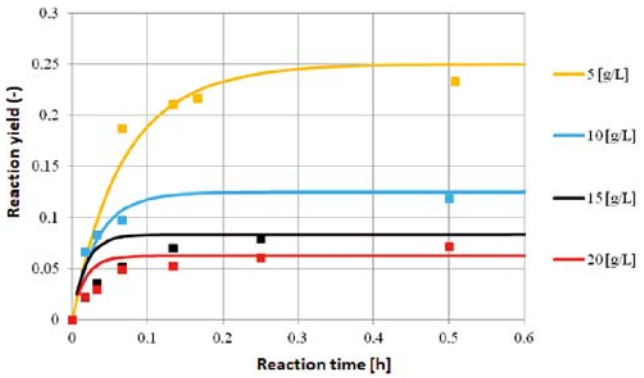

Figure 3. Dependence of the reaction yield from reaction time for the albumin-thermolysin system (60°C, pH 7.0, enzyme concentration 0.005 g/L, bovine serum albumin 5–20 g/L); points – experimental data; lines – model calculations

Kinetics of bovine serum albumin hydrolysis by pepsin

It has been experimentally proven that it is reaction of 1st order with competitive inhibition by products – a short-chain peptides (m<5)[15]. Kinetics (temp: 37°C, pH 2.0, enzyme concentration 0.005 [g/L]) is described by:

$$\frac{dc_i}{dt} = \frac{k}{1 + \frac{c_{inh}}{K_{inh}}}\left(\sum_{j=i+1}^{N} c_j \cdot (\beta_{j,i} + \beta_{j,j-1}) - c_i \cdot \sum_{m=1}^{i-1} \beta_{i,m}\right) \quad (6)$$

For this enzyme the chain of the bovine serum albumin consists of 71 reactive bonds (resulting in the presence of 2556 reagents) which may be divided into the three groups. As a result of the experimental data regression analysis (Fig. 4), values of the reactivity coefficient were obtained as follows: 9 bonds with β =1.0, 42 bonds with β = 0.24, 20 bonds with β = 0.0075, k = 2.94 [1/h]) and K_{inh} = 0.049 [g/L].

MODELLING PART

The effect of membranes with different selectivity was analyzed further. For clarity analysis, the coefficient R*, instead of the R coefficient (Eq. 3) was introduced. Its value is related to the smallest oligomer retained by a membrane. E.g. R* = 40 means that peptides containing 40 amino acids are retained by the membrane. Similarly, all other compounds with an amount of amino acids greater than 40 are retained. Peptides with a smaller amount freely pass through the membrane (a sharp membrane cut-off point was assumed).

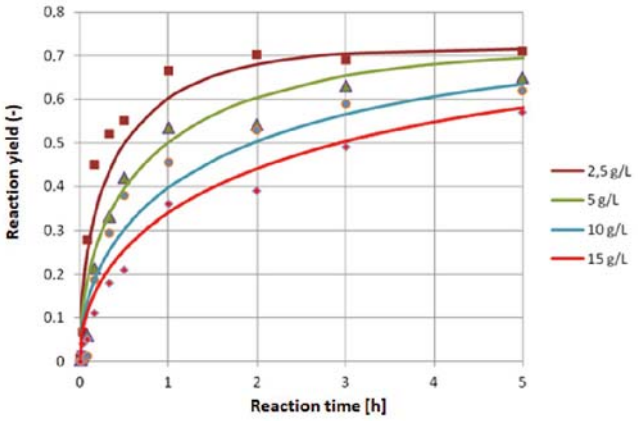

Figure 4. Dependence of the reaction yield from reaction time for the albumin-pepsin system (37°C, pH 2.0, enzyme concentration 0.005 g/L, bovine serum albumin 2.5–15 g/L); points – experimental data; lines – model calculations

According to the balance of the membrane reactor, the total mass of all components in the outlet stream is the same as in the inlet stream. While the membrane retains long chain oligomers, only short chain oligomers are present in the outlet stream. Therefore, their concentration will be higher than what would be in a classic flow reactor. This is very important for a reaction with autoinhibition. Assuming that the inhibitor is a low molecular weight oligomer, its concentration in the reaction zone is equal to the concentration in the stream leaving the reactor, i.e. higher than in the classic reactor. It has an influence on a given reaction kinetics.

Bovine serum albumin – subtilisin system

This system is characterized by the simplest kinetics, i.e. the first order without any inhibition. For this enzyme the longest chain without any reactive bonds (the biggest final product) contains 37 amino acids. The reaction yield in membrane reactor with the membrane selectivity of R^* equal to 40, 60, 80 was analyzed – Fig. 5.

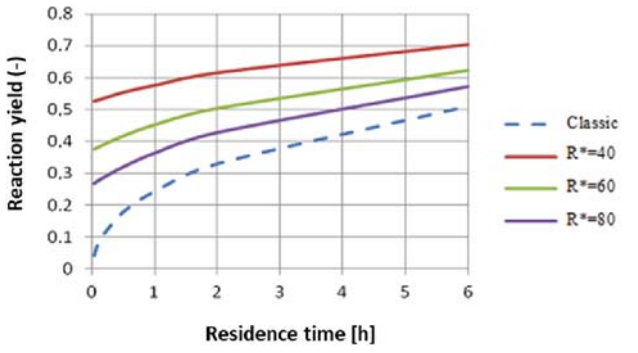

Figure 5. Reaction yield as a function of residence time, in a classical reactor and in a membrane reactor at different value of R* (50°C, pH 7.0, subtilisin concentration 0.01 g/L, bovine serum albumin concentration 15 g/L)

The higher values of the reaction yield were obtained in the membrane reactor due to the fact that in the outflow stream there were only short-chain reagents containing lower amount of unreacted bonds. The re-

action yield is higher when the membrane with lower value of R^* is used. This is particularly evident for very short residence times.

A membrane retaining the reagents causes an increase of their concentration in the reaction volume. Fig. 6 presents these values, while in the classical reactor their concentration is constant, equal to 15 g/L. As can be noticed in Fig. 5, by the membrane application it is possible to reach very high reaction yields at short residence times. However, for short residence times, there may occur unrealistic requirements for the concentration values in the reaction zone (a few hundreds g/L), hence both the membrane selectivity (R*) and the residence time must be precisely selected.

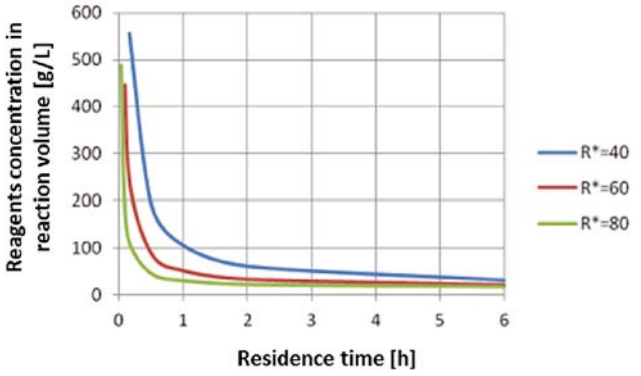

Figure 6. Influence of membrane selectivity (R*= 40–80) on reagents concentration in reaction volume at different residence times

Bovine serum albumin-thermolysin system

A non-competitive inhibition was shown for this reaction system. Using thermolysin to hydrolysis bovine serum albumin the longest chain without any reactive bonds contains 39 amino acids. The reaction yield in the membrane reactor with the membrane selectivity of R^* equal to 40, 50, 60 was analyzed – Fig. 7. For comparison, the dashed line shows the reaction yield rate in a classical reactor.

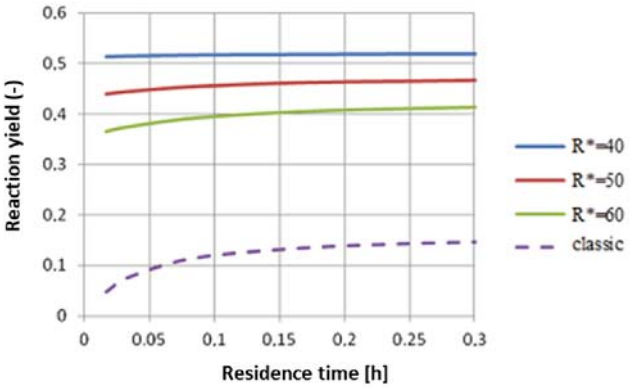

Figure 7. Reaction yield as a function of residence time, in a classical reactor and in membrane reactor at different value of R* (60°C, pH 7.0, thermolysin concentration 0.005 g/L, bovine serum albumin concentration 2 g/L)

In this case, the increase in reaction yield is very pronounced and it is very poorly dependent on the residence time.

There is an interesting phenomenon in this type of reaction kinetics. High value of membrane selectivity causes high concentration of reagents in the reaction volume. At the same time the high values of reaction yield cause high concentration of short peptides in the outflow stream. For compounds with R=0, the concentration in the reaction volume is the same as in the outflow stream (according Eq. (3)). At a certain concentration of biopolymer in the feed stream, the steady state can not be achieved. This is due to the fact that the high concentration of substrate implies a high concentration of inhibitors that completely stop the reaction.

Fig. 8 shows the values of the inhibitor concentration depending on the residence time and membrane selectivity at substrate concentration in the feed stream equal to 2 g/L. According to the equation (5), the concentration c_{inh} = 0.0145 [g/L] stops the reaction. The presented results are close to this value. For the membrane R^* = 40 and the albumin concentration a little higher (2.5 g/L), the steady state can not be achieved.

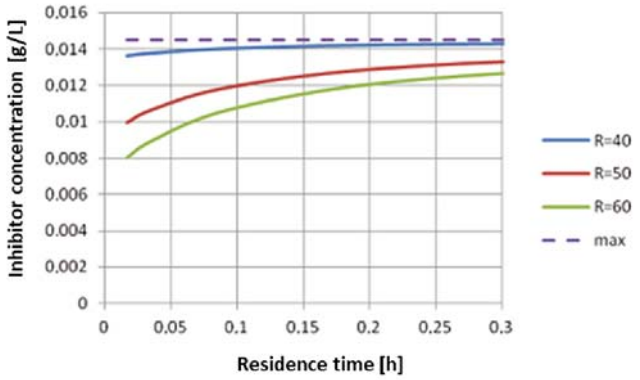

Figure 8. Inhibitor concentrations as a function of residence time in a membrane reactor at different value of R* (60°C, pH 7.0, thermolysin concentration 0.005 g/L, bovine serum albumin concentration 2 g/L)

Bovine serum albumin-pepsin system

A competitive inhibition, which depends on the substrate and inhibitor concentration ratio was shown for this reaction system.

Using pepsin to hydrolyze bovine serum albumin the longest chain without any reactive bonds contains 23 amino acids. The reaction yield in the membrane reactor with the membrane selectivity of R^* equal to 25 and 40 was analyzed – Fig. 9.

In this type of inhibition, there is no limitation in the concentration of biopolymer in the feed stream. The effect of membrane bioreactor is similar like in the case of subtilisin (the first order reaction).

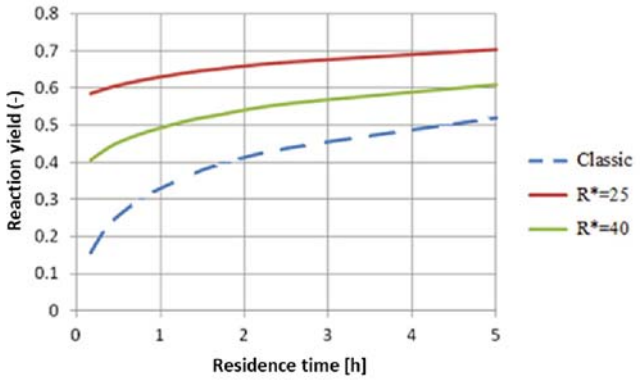

Figure 9. Reaction yield as a function of residence time, in a classical reactor and in membrane reactor at different value of R* (37°C, pH 2.0, pepsin concentration 0.005 g/L, bovine serum albumin concentration 5 g/L)

SUMMARY AND CONCLUSIONS

The reaction of enzymatic depolymerization of protein taking place in the membrane reactor according to the three different types of kinetics was analyzed. It was found that regardless of type of kinetics, the reaction yield in the membrane reactor is clearly higher than in the classic flow reactor. The analysis of the process allows to derive following conclusions:

1. The more selective membrane used, the higher reaction yields are obtained. However, it should be remembered that the applied membrane must allow the final products (also the chains containing a few dozen mers) to leave the reaction volume.

2. The strongest effect of the use of the membrane reactor is obtained for autoinhibition reactions, especially for non-competitive ones. For such reactions, the reaction yield is characterized by a weak dependence on the residence time, and high values are already obtained for very short times.

3. The effect of increasing the reaction yield results from the increased total concentration of reagents in the reaction volume. For membranes with very high selectivity, this concentration can reach an unrealistic value.

4. In the case of reactions with non-competitive autoinhibition, for a given membrane a limit of polymer concentration in the inlet stream above exists which the reaction stops and it is impossible to obtain a steady state.

Nomenclature

c – concentration, g/L
k – kinetic constant, 1/h
K_{inh} – inhibition constant, g/L
Q – volumetric stream, L/h
R, R^* – rejection coefficient
r – reaction rate, g/(L·h)
t – reaction time, h
V_R – reactor volume, L

β – reactivity coefficient
τ – residence time, h

indexs:

inh – inhibitor
i,j,k – position in olygomers chains
m – mer (amino acid)

ACKNOWLEDGEMENTS

Project supported by Wroclaw Centre of Biotechnology, The Leading National Research Centre (KNOW) program for years 2014–2018 and by NCN project No. 2011/03/B/ST8/06029.

LITERATURE CITED

1. Mahammad, S., Prud'homme, R.K., Roberts, G.R. & Khan, S.A. (2006). Kinetics of enzymatic depolymerization of guar galactomannan. *Biomacromolecules 7*(9), 2583–2590. DOI: 10.1021/bm060333+.

2. Muzzarelli, R.A.A, Stanic, V. & Ramos, V. (1998). *Enzymatic depolymerization of chitins and chitosans*. In: Methods in Biotechnology, Vol. 10: Carbohydrate Biotechnology Protocols, Ed. C. Bucke © Humana Press Inc., NJ.

3. Slominska, L., Grajek, W., Grzeskowiak, A. & Gocalek, M. (1998). Enzymatic starch saccharification in an ultrafiltration membrane reactor. *Starch 50*(9), 390–396. DOI: 0038-9056/98/0909-0390$17.50+.50/0.

4. Trusek-Holownia, A. & Noworyta, A. (2000). Dipeptide enzymatic synthesis in a two-phase membrane reactor. *Chem. Pap. 54*(6B), 442–447.

5. Nguyenhuynh, T., Nithyanandam, R., Hwa Chong, Ch. &Krishnaiah, D. (2017). A review on using membrane reactors in enzymatic hydrolysis of cellulose, *J. Eng. Sci. Technol. 12*(4), 1129–1152.

6. Hang, H., Bao, S., Zhao, M., Wang, B., Zhou, S. & Jiang, B. (2015), Enzyme membrane reactor coupled with nanofiltration membrane process for difructose anhydride III from inulin conversion. *Chem. Eng. J. 276*, 75–82. DOI: 10.1016/j.cej.2015.04.018.

7. Olano-Martin, E., Mountzouris, K.C., Gibson, G.R. & Rastall, R.A. (2001). Continuous production of pectic oligosaccharides in an enzyme membrane reactor, *J. Food Sci. 66* (7), 966–971. DOI: 10.1111/j.1365-2621.2001.tb08220.x.

8. Marquez M.C. & Vazquez M.A. (1999). Modeling of enzymatic protein hydrolysis," *Proc. Biochem. 35*, 111–117. DOI: 10.1016/S0032-9592(99)00041-2.

9. Fernandez, A. & Riera F. (2013). β-Lactoglobulin tryptic digestion: a model approach for peptide release. *Biochem. Eng. J. 70*, 88–96. DOI: 10.1016/j.bej.2012.10.001.

10. Rawlings, N.D., Barrett, A.J. & Bateman, A. (2012). MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res. 40*, 343–350. DOI: 10.1093/nar/gkr987.

11. Trusek-Holownia, A. & Noworyta, A. (2015). A model of kinetics of the enzymatic hydrolysis of biopolymers - a concept for determination of hydrolysate composition. *Chem. Eng. Proc. 89*, 54–61. DOI: 10.1016/j.cep.2015.01.008.

12. Orecki, A. & Tomaszewska, M. (2007) The oily wastewater treatment using the nanofiltration process. *Pol. J. Chem. Technol*. 9 (4), 40–42. DOI: 10.2478/v10026-007-0086-8.

13. Trusek-Holownia, A., Przybyl, A. & Noworyta, A. (2014). Zagospodarowanie odpadowej serwatki w kierunku aktywnych peptydow (in Polish). *Inz. Ap. Chem.* 53 (4), 314–315.

14. Trusek-Holownia, A., Lech, M. & Noworyta, A. (2016). Protein enzymatic hydrolysis integrated with ultrafiltration: Thermolysin application in obtaining peptides. *Chem. Eng. J. 305*, 61–68. DOI: 10.106/j.cej.2016.05.087.

15. Labus, K., Trusek-Holownia, A. & Noworyta, A. (2015), Kinetics of protein hydrolysis catalyzed by pepsin. In: 42nd International Conference of Slovak Society of Chemical Engineering proceedings, Tatranské Matliare, Slovakia, May 25–29, 2015. Ed. Jozef Markoš, Slovakia, 465–472.