INFLUENCE OF PERMEABILIZATION ON THE CATALYTIC ACTIVITY OF YEAST CELLS *YARROWIA LIPOLYTICA* KKP 379 IN THE SYNTHESIS OF PLASTICIZER – DIOCTYL ADIPATE

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Summary. Plasticizers are among the most important additives required for processing of polymer materials. In this paper, biotechnological synthesis of adipic plasticizer – dioctyl adipate was presented with the participation of yeast biomass of *Yarrowia lipolytica* KKP 379. The reaction was based on transesterification of dimethyl adipate with octan-1-ol. Relative to the whole-cell catalyst were used both physical (treated with glass beads) and chemical (use of isooctane and Triton X-100) methods of permeabilization. The research confirmed the possibility of the synthesis of dioctyl adipate catalyzed by whole cell yeast with the yield 60% (58% ±1,8%). The permeabilization techniques used did not contribute to increasing the reaction efficiency. Among the methods of violating the continuity of the yeast cell wall, the chemical method using Triton X-100 is of interest. Approximately 50% conversion was achieved in this reaction and no decrease in yield was observed during 96 hours of reaction, as opposed to other methods permeabilization.

Key words: plasticizers, dioctyl adipate, *Yarrowia lipolytica*, permeabilisation

INTRODUCTION

Plastics play an important role in every area of our life. Their widespread use is associated with a number of features and properties which cannot often be obtained with natural materials. Plasticizers are an important class of low molecular weight non-volatile compounds that are widely used in polymer industries as additives. There are derivatives of dicarboxylic acids, e.g. phthalic, adipic, sebacic or maleic acid. The primary role of such substances is to improve the flexibility, plasticity and processing of polymers,

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which is accomplished by reducing temperature of second-order phase transition and glass transition temperature (Tg) [Rosen 1993, Jia et al. 2018]. It is possible due to the penetration of plasticizer molecules (with low molecular weight) between the chains or agglomerates of plastic macromolecules, which results in a decrease in intermolecular interactions (reduction of van der Waals forces) and in an increase in distance between plastic segments. This translates into increased system flexibility – the plastic is then softer and easier to form [Rahman and Brazel 2004, Pradhan et al. 2007].

At present, the industrial production of plasticizers is dominated mainly by chemical synthesis, which has an adverse effect on the natural environment [Abrishami et al. 2018, Chizari and Bayat 2019, Shen and Kwon 2019].

Due to the growing interest and concern for environmental protection, the plastics industry, using the wide possibilities of genetic engineering, undertakes pilot attempts to synthesize plasticizers by enzymatic means [Tian et al. 2016, Andriukonis et al. 2018]. Due to the relatively high cost of commercial enzyme preparations, research laboratories are trying to synthesize plasticizers with the use of whole-cell catalysts (whole cell system catalysts). Since most plasticizers are esters, in the synthesis based on esterification or transesterification reactions there are most often used whole cells of microorganisms capable of producing lipolytic enzymes that catalyze the above-mentioned types of reactions. Among microorganisms, yeast from the *Yarrowia lipolytica* species is a well-known and increasingly used producer of lipases. They are very popular, not only due to the non-pathogenicity or ease of their reproduction, but also due to showing interesting physiological, metabolic and genetic features. *Yarrowia lipolytica* synthesizes both extracellular and intracellular lipases [Pereira-Meirelles et al. 2000, Krzyczkowska and Białecka-Florjańczyk 2011].

Relying on the intracellular lipolytic activity of *Yarrowia* yeast cells in enzymatic catalysis allows to avoid the complicated and expensive process of isolating and purifying the enzyme. However, one should keep in mind that the negative side of using whole-cell systems is the barrier which the cell wall forms for contact of the enzyme with the substrate. Hence, often in order to facilitate the access of the biocatalyst to the reactants, permeabilization methods of the cell wall are used which also enable to increase its permeability. Permeabilization techniques aimed at violating the continuity of the wall structure and cell membranes are based on biological (enzymatic), chemical or physical methods. The latter are among the cheapest ways of cell disintegration, but unfortunately they can cause denaturation of the enzyme protein. Chemical methods are based on the use of organic solvents, e.g. isooctane, acetone; surfactants e.g. Tween 20, 80, Triton X-100, SDS; chelating compounds, e.g. EDTA or cysteine solution, which reduces the disulfide bridges of cell wall proteins. These chemical agents allow permeabilization by reducing the content of phospholipids in the cell membrane, as a result of which pores are formed, enabling passive transport of small molecules - substrates and reaction products [Naglak et al. 1990, Somkuti et al. 1998].

This work aimed to assess the effect of permeabilization of *Yarrowia lipolytica* KKP 379 yeast cells on their catalytic activity in the model synthesis reaction of the plasticizer - dioctyl adipate. Both physical (glass beads treatment) and chemical (using isooctane and Triton X-100) permeabilization methods were used for the whole cell catalyst.

MATERIAL AND METHODS

The research material used in the experiments was the strain of yeast *Yarrowia lipolytica* KKP 379, derived from the Collection of Industrial Microbial Cultures of the Institute of Agricultural and Food Biotechnology in Warsaw.

Culture conditions of *Yarrowia lipolytica* KKP 379. In the first stage, an inoculum was prepared. The strain of yeast *Yarrowia lipolytica* KKP 379 was transplanted from the agar slopes into a flat-bottomed flask containing 100 cm^3 of YPG medium (medium composition: yeast extract 10 $g \cdot dm^{-3}$, peptone 20 $g \cdot dm^{-3}$, glucose 20 $g \cdot dm^{-3}$). Culturing was carried out for 24 hours at 28°C with shaking at 140 rpm. The second stage was the cultivation of *Yarrowia lipolytica* yeast biomass in the bioreactor BIOFLO 3000 (New Brunswick Scientific Edison, N.I., USA). Yeast was propagated in a volume of 4 dm^3 YPG medium with 1% addition of extra virgin olive oil (La Pedriza), which served as a lipase inducer [Zarevúcká 2012]. The earlier multiplied inoculum constituted 0.025% of the bioreactor working volume. The culture was carried out for 72 hours at 28°C with a mixing speed of 300 rpm and the oxygenation 0.2 vvm (volume air per cultivation volume and minute). After completion of the culture, the yeast biomass was separated from the culture liquid by centrifugation (5000 rpm).

Dioctyl adipate synthesis reaction. The synthesis of dioctyl adipate (Fig. 1) was based on the transesterification reaction of dimethyl adipate with octane-1-ol in a solvent-free environment. A two-fold molar excess of alcohol was used relative to the diester. The reaction catalyst was *Yarrowia lipolytica* KKP 379 yeast biomass, used in an amount of about 10–15 g (the quantity of biomass standardized based on its lipolytic activity in spectrophotometric measurement of *p*-nitrophenyl laurate hydrolysis). The reaction was carried out in 100 cm^3 round-bottomed flasks at 40°C for 96 hours. To ensure even mixing throughout the volume, mechanical stirrer was used. Due to the methanol formed as a result of the transesterification reaction, the flasks with the reaction mixture were open to enable the alcohol to evaporate. Molecular sieves (4A) were used to remove water released from the cellular space during the reaction and were added in the amount of 10 g. During the reaction samples were taken from reaction mixture every 24 hours for chromatographic analysis. Prior to analysis, the samples were appropriately diluted using methylene chloride.
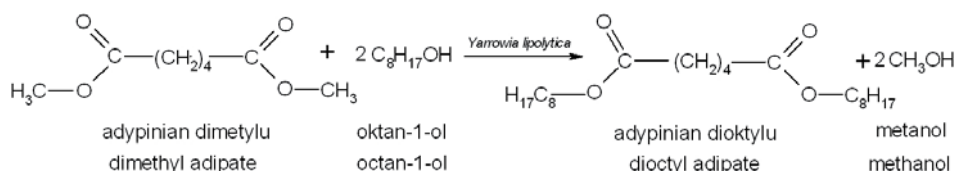

Fig. 1.   Reaction the dioctyl adipate synthesis catalyzed by *Yarrowia lipolytica* KKP 379 yeast

Rys. 1.   Reakcja syntezy adypinianu dioktylu katalizowanego przez drożdże *Yarrowia lipolytica* KKP 379

Permeabilization of *Yarrowia lipolytica* KKP 379 yeast cells. Yeast cells were permeabilized by pretreating the catalyst with glass beads, isooctane and an aqueous solution of Triton X-100 surfactant. Permeabilization conditions:

•   Glass beads – centrifugation of thawed biomass in a centrifuge with glass beads for 10 minutes at 5000 rpm;
•   Isooctane – 30-minute incubation of biomass in isooctane at room temperature;
•   Triton X-100 – 30-minute incubation of biomass with 5% aqueous surfactant solution at room temperature.

Chromatographic analysis. Samples of the post-reaction mixture were analyzed using GC gas chromatography – YL 6100 Young Lin Instrument chromatograph with a BPX capillary column (30 m x 0.25 mm) and a flame ionization detector (280ºC), with azote as a gas vector, at a flow rate of 1.1 ml/min. The analysis program was as follows: the initial temperature was 100ºC, the final temperature was 230ºC, the temperature increase was $10ºC·1\ min^{-1}$, finally it was kept at 230ºC for 10 min. The total analysis time was 23 minutes. Retention times of individual components of the mixture were: approx. 5.5 min – octanol, approx. 9.5 min – dimethyl adipate, approx. 13 min – monoester (adipic acid substituted by one carboxyl group with methanol and by the other with octanol), approx. 16.5 min – diester (dioctyl adipate).

Statistical methods. The obtained results were processed statistically using the program Statgraphics Plus 4.1. The significance of differences between the mean values from individual experiments was assessed using one-way analysis of variance (ANOVA) with the significance level $\alpha = 0.05$.

RESULTS AND DISCUSSION

Assessment of the catalytic activity the biomass of yeast *Yarrowia lipolytica* KKP 379 (before and after permeabilization) it was based on the synthesis reaction of dioctyl adipate (DOA) – a plasticizer added to polyvinyl chloride, nitrocellulose, ethyl cellulose and most synthetic rubbers. The research was aimed at developing DOA biosynthesis which in combination with other plasticizers, such as dioctyl phthalate (DOP) or dibutyl phthalate (DBP), is widely used in the food industry – for the production of cold-resistant thin films or membrane packaging [Rahman and Brazel 2004].

In preliminary studies, DOA synthesis was carried out with the participation of "raw" (before permeabilization) yeast biomass *Yarrowia lipolytica* KKP 379. The lipolytic activity of the used yeast biomass was on average around 1.73 ±0.22 U/g. The collected results (Fig. 2) allowed to observe that the use of whole-cell catalyst allowed the synthesis of plasticizer with a maximum yield of 58.4% ±1.8%. The highest level of conversion was observed on the third day of reaction. After this time, reaction efficiency dropped to 31.3% ±6.3% after 96 hours.

Since the dioctyl adipate synthesis reaction was carried out on the basis of transesterification, assuming that it will be irreversible, it is believed that the observed decrease in yield should not be associated with the reversal of the reaction. It is assumed that some
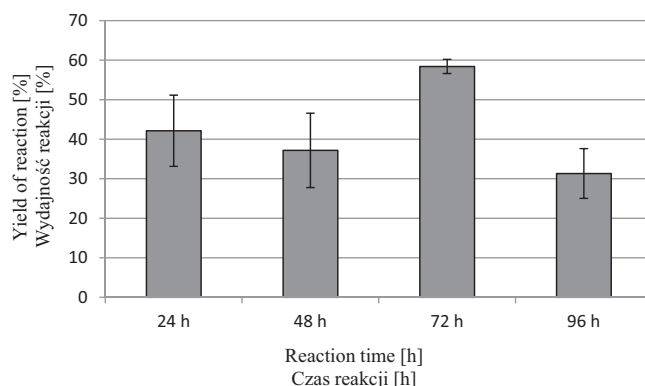
Fig 2.   The yield of dioctyl adipate synthesis catalyzed by whole-cells *Yarrowia lipolytica* KKP 379 (before permeabilisation)

Rys. 2.   Wydajność reakcji syntezy adypinianu dioktylu katalizowanej przez komórki drożdży *Yarrowia lipolytica* KKP 379 nie poddane permeabilizacji

of the plasticizer molecules, after three days of reaction, may be hydrolyzed by active lipolytic enzymes present in yeast cells. The natural function of these enzymes is the hydrolysis of triacylglycerols to glycerol and free fatty acids, which can contribute to the breakdown of long-chain products. It is also likely that some of the plasticizer molecules undergo adhesion to the surface of the microorganism cells after some time, which may result from the difference in surface tension, and which contributes to the observed decrease in reaction efficiency. Aguedo et al. [2004] shows the adhesion of hydrocarbons to the cell of *Yarrowia lipolytica* in their research. Carrying out the biotransformation of methyl ricinoleate to gamma-decalactone, the authors conducted a MATH test (Microbial Adhesion to Hydrocarbons) proving the adhesion of the decane particles to the yeast cells.

Because in the above tests the maximum yield reached slightly over 55% (58.4 ±1.8%), attempts were made to increase the catalytic activity of the biocatalyst by using physical and chemical permeabilization techniques. It was assumed that by increasing the permeability of the yeast cell wall, the contact between enzymes accumulated in the periplasmic space, those associated with the cell wall and concentrated in the cytoplasm, would be facilitated. So far published data on the lipolytic activity of microorganisms indicate that about 70.5% of intracellular lipases are connected with the cell wall, 13% are in the cell nucleus, 11% in microsomes (fraction of cell homogenate), about 3% are present in mitochondria and about 2.5% in cytosol [Adamczak 2003].

Yeast biomass after permeabilization with glass beads and using chemical methods – isooctane and Triton X-100 (Fig. 3) was evaluated based on catalytic activity in the synthesis reaction of dioctyl adipate. In addition to physical permeabilization, it was decided to check the effectiveness of chemical methods due to the fact of the hydrophilic-hydrophobic balance, which is established in the enzyme molecule due to the action of an organic solvent, and which ensures an increase in the affinity of the enzyme for the substrate, and thus an increase in its catalytic activity. It has been scientifically

proven that treatment with organic solvent leads to quite large conformational changes in lipases, which from the closed form (active place hidden under a hydrophobic lid) change into an open (lid shift) by keeping most of the hydrophobic amino acids on the catalyst surface [Zaman et al. 2005]. Numerous literature reports indicate that the most effective solvents that have a beneficial effect on the catalytic activity of enzymes are: heptane, hexane and isooctane [Yadav et Trivedi 2003, Villeneuve 2007]. In transesterification vinyl acetate with *n*-octanol, with the participation of commercial lipase Novozym 435, the highest conversion of substrates was achieved in the presence of *n*-heptane [Yadav et Trivedi 2003]. According to the authors, in the synthesis of esters, more hydrophobic solvents are preferred, which allow to maintain the aqueous layer around the enzyme, which is necessary to maintain its catalytic activity. The high biocatalytic activity in the presence of hydrophobic solvents is also confirmed by Akoh and Yee [1998]. In the synthesis of: geranyl acetate and citronellol acetate carried out using, among others: chloroform, benzene, toluene, cyclohexane, hexane, heptane and isooctane, the highest yields were obtained in the presence of the last three solvents. During research on the synthesis of dioctyl adipate, it was decided to permeabilize cells with isooctane. Promising results in this area of research were received by Wang et al. [2007], who proved that the action of isooctane can contribute to even a 39% increase in the synthetic activity of enzymes (while the use of e.g. acetone allows an increase in the level of catalytic activity by a maximum of about 30%).

However, the data collected in Figure 3 showed the negative impact of permeabilization, regardless of the method used. In the case of the reaction carried out with the participation of cells centrifuged with glass beads, the maximum yield of plasticizer synthesis reached only the level of 37.6 ±3.5% (the second day of the reaction) and was about 20% lower compared to the reaction carried out with the participation of "raw" biomass (Fig. 2 – maximum efficiency when using raw biomass was 58.4 ±1.8%). Based on the collected results, it is assumed that perhaps due to incorrectly selected parameters (e.g. too high centrifugation speed) the yeast cell wall disintegrated, the continuity of cell wall structures and membranes was broken, which resulted in the release of the cell content into the external environment. The consequence of this was probably the removal of some lipases together with the supernatant formed after centrifugation of the biomass with beads, which resulted in lower catalytic activity and lower reaction efficiency. The impact of the method and parameters of the permeabilization process on properties, including the activity of the biocatalyst obtained, is confirmed among others by Mason [2003] and Borthwick et al. [2005].

Water activity could also play a significant role in the level of achieved reaction efficiency. Literature data indicate that in reactions involving lipases in the environment of organic solvents, a small amount of water is always necessary as it determines the maintenance of the three-dimensional structure of the enzyme, protects its catalytic form and affects thermal stability. Optimal water activity ensuring the proper course of esterification or transesterification in lipase-catalyzed reactions should be between 0.25 and 0.45, which corresponds to 0.5–1% of water content in the reaction environment [Villeneuve 2007]. The optimal water content in the reaction medium during the synthesis of 2-ethylhexyl fatty acid esters with the participation of the immobilized lipase of *Candida sp*. is also mentioned by the team of He et al. [2002]. According to the authors' research,
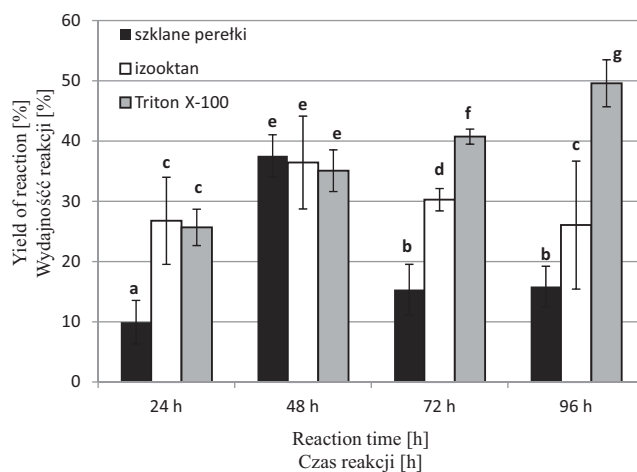
mean values in the rows with different superscripts are signifi cantly different at $\alpha = 0{,}05$

wartości średnie oznaczone różnymi literami różnią się statystycznie istotnie na poziomie $\alpha = 0{,}05$

Fig. 3.   The yield of dioctyl adipate synthesis catalysed by whole-cells *Yarrowia lipolytica* KKP 379 after permeabilization

Rys. 3.   Wydajność reakcji syntezy adypinianu dioktylu katalizowanej przez biomasę drożdży Yarrowia lipolytica KKP 379 poddanej permeabilizacji

the level of water present in the synthesis processes ensuring high performance should be within 0.6% (v/v). This is also confirmed by the synthesis of the plasticizer – dibutyl adipate with the participation of CAL B lipase, which proceeded with the highest efficiency at 0.53% (v/v) water content [Tanino et al. 2009]. In case of experiments carried out by our team, the disintegration of the yeast cell wall as a result of centrifugation with glass beads could contribute to the release of significant amounts of water from the inside of the yeast cells, which resulted in an increase in water activity and could negatively affect the enzyme.

The treatment of *Yarrowia lipolytica* cells with isooctane was slightly better compared to experiments with glass beads, where maximum conversion was achieved at the level of approx. 40% (36.4 ±7.7%) in 48 hours of synthesis. During the 96-hour transesterification reaction, taking into account the standard deviation of the measurements, the yield remained at a similar level – in 96 hours 26.1 ±10.6%. The results showing the negative impact of yeast biomass treatment with isooctane (compared to the "raw" catalyst – Fig. 2) are different from the data obtained by our team in the synthesis of the fragrance compound – 2-phenylethyl acetate [Białecka-Florjańczyk et al. 2012]. In the synthesis of this ester, also with the participation of *Yarrowia* yeast cells, we conducted 30- and 60-minute permeabilization with isooctane. The treatment with organic solvent, in the experiment mentioned above, had a positive impact on the level of synthesis reaction, the yield increased from about 30% (for non-permeabilized cells) to 90% (after 60 minute of isooctane treatment). However, it should be remembered that the achieved level of reac-

tion efficiency is significantly influenced not only by the catalyst used, but also by the type of catalyzed reaction and the type of substrates used.

The reduced catalytic activity of permeabilized *Yarrowia* yeast biomass can also be caused by the solvent incubation conditions at room temperature. The temperature in the range of 23–25ºC contributes to the activity of proteolytic enzymes that may have been released from the inside of the cell as a result of permeabilization, and which have a degrading effect on lipases. The relationship between lipolytic activity and the presence of proteolytic enzymes has been noticed and confirmed by immunoenzymatic studies in case of the heterologous lipase B expression system of *Candida antarctica* and the cellulose binding molecule of *Neocallimastix patriciarum* in *Pichia pastoris* Mut + [Jahic et al. 2003]. Considerations on extracellular lipolytic activity conducted by scientists confirmed that the reduction of protease activity due to modification of the pH of the medium and temperature resulted in even a twofold increase in the level of lipases in the supernatant compared to the culture under standard conditions.

When Triton X-100 nonionic surfactant was used in permeabilization, the synthesis yields were also lower than those obtained with using "raw" biomass. The maximum efficiency reached the level of about 50% (49.6% ±3.9%) after 96 h of reaction (Fig. 3). In contrast to the previously discussed results, in this reaction, comparing the level of conversion between individual reaction methods, no decrease in the yield of synthesis was observed, which seems promising in the perspective of extending the permeabilization time. It can be observed that the reaction using Triton X-100 was about 22% more effective than those using the catalyst after treatment with isooctane or glass pearls. Perhaps Triton X-100 more effectively dissolve lipids from cell membranes, due to which their permeability is higher, and thus the interaction of the enzyme with the substrate is better [Jamur and Oliver 2009].

CONCLUSIONS

Yarrowia lipolytica KKP 379 yeast biomass can be an effective catalyst for the synthesis of dioctyl adipate, performed by transesterification of dimethyl adipate with octane-1-ol. The use of whole-cell catalyst allows the reaction to be carried out with an efficiency of approx. 60% (58.4% ±1.8%). The use of physical and chemical methods of permeabilization of the yeast cell wall contributes to the reduction of reaction efficiency. Among the permeabilization techniques used (glass beads, treatment with isooctane or Triton X-100), assuming that the synthesis reaction time is extended, a chemical method with the participation of the non-ionic surfactant Triton X-100 seems promising. The synthesis of dioctyl adipate catalyzed by permeabilized biomass proceeds then with a yield of approximately 50% (49.6 ±3.9%) and is 22% more effective than the reaction using a catalyst after treatment with glass beads or isooctane.

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WPŁYW PERMEABILIZACJI NA AKTYWNOŚĆ KATALITYCZNĄ KOMÓREK DROŻDŻY *YARROWIA LIPOLYTICA* KKP 379 W SYNTEZIE PLASTYFIKATORA – ADYPINIANU DIOKTYLU

Streszczenie. Plastyfikatory to związki organiczne stanowiące podstawowy środek pomocniczy w poprawie elastyczności i przetwórstwa polimerów. Są one pochodnymi kwasów dikarboksylowych, najczęściej: kwasu ftalowego, sebacynowego, maleinowego oraz adypinowego. Estry kwasu adypinowego mają szerokie zastosowanie m.in. w przemyśle spożywczym, farmakologicznym i motoryzacyjnym. W pracy podjęto próbę syntezy plastyfikatora z grupy adypinianów – adypinianu dioktylu na drodze transestryfikacji adypinianu dimetylu oktan-1-olem przy zastosowaniu biomasy drożdży *Yarrowia lipolytica* KKP 379. W stosunku do katalizatora całokomórkowego zastosowano zarówno fizyczne (traktowanie szklanymi perełkami), jak i chemiczne (stosowanie izooktanu lub Tritonu X-100) metody permeabilizacji. Postęp reakcji śledzono chromatograficznie GC. Wyniki badań potwierdziły możliwość syntezy adypinianu dioktylu katalizowanej „surową" biomasą drożdży z wydajnością około 60% (58% ±1,8%). Zastosowane techniki permeabilizacji nie przyczyniły się do podwyższenia wydajności transestryfikacji. Spośród stosowanych metod naruszania ciągłości struktury ściany komórkowej zainteresowanie wzbudza metoda chemiczna, z zastosowaniem Tritonu X-100. Wydajność syntezy plastyfikatora z udziałem komórek poddanych tej technice permeabilizacji była wprawdzie o ok. 10% niższa (przereagowanie 49,6% ±3,9%) w stosunku do reakcji z zastosowaniem „surowej" biomasy, nie mniej jednak w przeciwieństwie do pozostałych stosowanych metod, nie obserwowano spadku wydajności reakcji w ciągu 96 godzin jej prowadzenia.