

Immobilization of a non-heme chloroperoxidase from *Serratia marcescens* by entrapment into Ca-alginate beads and in semi-permeable protein membranes

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Abstract: Chloroperoxidase (CPO) (EC 1.11.1.10) from *Serratia marcescens* W 250 was immobilized in Ca-alginate beads and semi-permeable membranes. The thermal stability, stability under storage and operational stability of the immobilized enzyme under determining halogenating and phosphatase activity have been reported. The addition of $[\text{Fe}(\text{CN})_6]^{3-}$ included into beads was found to increase the activity of the immobilized enzyme. Polyethylenimine(PEI)- and glutaraldehyde (GA) - coated beads showed good operational stability but the activity of the enzyme especially in the case of PEI greatly reduced. GA-coated beads with ferricyanide possess good stability under operational conditions and could be stored in the reaction buffer at 4°C for at least 5 weeks. The most applicable variant of semi-permeable membranes was made of gelatin as a protein carrier and treated with 0.5 % GA for 2 min. The best variants of immobilized CPO preparations for determining halogenating and phosphatase activity were proved in the fluidized-bed column bioreactor. The yield of products was 91 % for GA-coated gelatin membranes with monochlorodimedone (MCD) and phenol red as substrates at the flow rate through the reactor of 27 ml/hr and they lost only about 20% of activity during 10 days. For Ca-alginate beads the yield of tetrabromphenol and p-nitrophenol (pNP) was 63 % and 56 % correspondingly at 32 ml/ hour. For pNP the yield stays stable at such a level during 10 days of the reactor work.

Keywords: chloroperoxidase, immobilized enzyme, calcium alginate, semi-permeable membranes, halogenation, phosphatase activity, fluidized-bed bioreactor.

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Introduction

Haloperoxidases (CF 1.11.1.10) are considered to be the enzymes capable of catalyzing the formation of carbon-halogen bonds in the presence of hydrogen peroxide and halide ions (Cl^- , Br^- , I^-). A wide spectra of redox reactions such as epoxidation, N-demethylation, hydroxylation of aromatic systems, oxidation of indole, sulfides to sulfoxides, alcohols to aldehydes can also be proceeded with these catalyzing agents (Neidleman and Geigert, 1986). Therefore, haloperoxidases prove to be helpful in the field of organic synthesis and especially in the biotechnological sphere.

Whereas eucaryotic haloperoxidases are heme- or vanadium-containing, in bacteria nonheme halogenating enzymes have also

been detected (van Pee, 1996). Thus, halogen atom insertion takes place in the medium of acetate ions that bind covalently to a nucleophilic Ser residue of catalytic triad Asp-Hys-Ser to form an ester that is subsequently hydrolyzed with hydrogen peroxide to form peracetic acid. The last probably is the cause of large number of oxidation reactions that could be performed by this class of enzymes. Lately the investigation was carried out but did not confirm the suggestion that hydrolysis is non- enzymatic process and goes outside the active center (Emilie F. Gerard, 2023). We suggest here that phosphate ions could also esterize the active center that highly reminds the mechanism of hydrolysis by serine hydrolases. Additionally to their broad substrate specificity the bacterial nonheme haloperoxidases possess high biological activity

and very good thermostability under reaction conditions. At the same time they are quite available due to successful cloning of appropriate genes (Pfeifer et al, 1992, Pelletier et al, 1994). However, in the industrial production any type of enzymes can be hardly used without the possibility of its recycling. For these purposes immobilized biologically active substances have been applied recently. Immobilization of enzymes means fixation of a catalyzing agent on a solid, in any kind of gel or particular separation of an enzyme from reaction products by including into vesicles or semi-permeable membranes.

The immobilization of an enzyme by entrapment in Ca-alginate beads has certain advantages with regard to cost and equipment. However, the operational stability of immobilized enzymes prepared so far by this method is usually rather low because of the leakage of an enzyme and its activity during recycling is not always high enough (Nahalka et al, 2004). Any kinds of hardening and stabilizing reagents have been used to improve recyclability of immobilized in Ca-alginate beads enzymes (Lee K.H. et al, 1992). The advantage of semi-permeable membranes is a possibility to be used in many kinds of technical appliances such as enzyme electrodes or enzyme-containing purifying membranes. The aim of this work is to develop immobilized preparations of the chloroperoxidase from *Serratia marcescens* W 250 capable of transformation of organic substrates with possibility of recycling.

Materials and Methods

Enzyme and chemical reagents

The bacterial strain *Serratia marcescens* W250 was obtained and generously provided from the Institute of Hohenheim, Germany, by the group of Prof. K.-H. van Pee. Nonheme haloperoxidase was isolated and purified from *Serratia marcescens* W250 strain by the method having been described previously (Burd et al., 1996). It is highly purified and has a specific activity of 30 units mg^{-1} protein. The standard assay for halogenating activity is the bromination of MCD that is followed by decreasing of absorbency at 290 nm. One unit of enzyme activity is defined as the amount of the enzyme that catalyses the brominating of 1 μmol of monochlorodimedon (MCD) per min at pH 5.5 and at 25°C (Morris, 1966). MCD was purchased from Sigma. Calcium chloride, sodium alginate, polyethylenimine (PEI) and glutaraldehyde were purchased from Loba Finechemie and phenol red – from Serva (Germany). All chemicals were used without further purification.

Preparation of immobilized CPO

Entrapment in calcium alginate beads. 1 cm^3 haloperoxidase solution (0.05 mg cm^{-3}), in the case of beads with $\text{K}_3[\text{Fe}(\text{CN})_6]$ containing 0.01M potassium ferricyanide, was added to 1 cm^3 of 5% (w/v) sodium alginate solution. The mixture was stirred for 10 min and allowed to equilibrate for further 10 min. The mixture was then added dropwise into a gently stirred 20 cm^3 0.35 mol dm^{-3} CaCl_2 solution. Sphere- shape transparent beads (2-3 mm in diameter) were left in the CaCl_2 solution for 20 min. They were then allowed to harden in 0,2 mol dm^{-3} CaCl_2 solution for another 30 min. The beads were washed thoroughly with 0.1M sodium acetate (NaAc) buffer pH 5.5 containing 0.01M CaCl_2 and stored in it at 4°C until use (Woodward, 1988).

Modified entrapment in calcium alginate beads

The calcium alginate beads entrapping the enzyme prepared as described above were suspended in 100 cm^3 0.5 % (w/v) PEI or GA containing 0.2 mol dm^{-3} CaCl_2 solution (pH 6) for 5 min with stirring at room temperature. The beads were washed and stored as described before.

Entrapment in semi-permeable protein membranes

The CPO preparation used for immobilization in protein membranes was purified in K-phosphate buffer as described in (Preobrazhenskaya et al, 2003). After gel-filtration the highly-purified enzyme contained 48 $\mu\text{g/ml}$ of protein in 0.1 M phosphate buffer, pH 5.5 and had phosphatase activity of 0.164 Units per mg of protein.

For halogenating activity investigation the CPO immobilized in BSA, gelatin, RNAase or Cyt C membranes was prepared. When the phosphatase activity of CPO being investigated BSA and gelatin membranes were used. In the case of gelatin membranes gelatin was solubilized in the enzyme preparation containing 0.1 Units of CPO at 45°C till final concentration of 3%. Before becoming cold the solution was spread onto a piece of cellophane and dried at 25°C for 2 hours (Kolis et al, 1987). Then it was immersed for cross-linking in GA solution of appropriate concentration in 0.1 M phosphate buffer, pH 5.5 for 2 min or 3 min.

In the case of membranes made of BSA, RNAase and Cyt C the proteins were dissolved in the enzyme solution till final concentration 3 % and the appropriate amount of GA was added following the solution obtained was spread onto cellophane. The cellophane film of 3.85 cm^2 and 31.25 mm thick was treated with 1 M NaOH before use for improving hydrophilicity. The membrane with immobilized CPO obtained was left for 2 hours for hardening then washed three times with 0.1M phosphate buffer pH 5.5.

Activity of free and immobilized CPO

Halogenating activity determination

The substrate contained phenol red (25 μM), hydrogen peroxide (7.2 mM), sodium bromide (0.45 M), sodium azide (8.92 mM) and 10 mM CaCl_2 in 1 M NaAc buffer. The reaction was initiated by adding 0.1 cm^3 of enzyme (0.07 mg cm^{-3}) dissolved in 0.1 M NaAc buffer pH 5.5 to 1 cm^3 of the substrate. The absorbency of the solution was measured spectrophotometrically at 590 nm at room temperature due to the increasing amount of bromophenol blue ($\epsilon = 100 \pm \text{mM}^{-1} \text{cm}^{-1}$ at 590 nm) (Neidلمان and Geigert, 1986; Preobrazhenskaya et al, 2000, Littlechild et al, 2009). The molar extinction coefficient for bromophenol blue was determined by us when the reaction of bromination of phenol red was fully completed. One unit of the brominating activity of CPO was defined as the amount of the enzyme required to liberate 1 μM of bromophenol blue per min at pH 5.5 at 20°C.

Halogenating activity of immobilized CPO

A reaction mixture composed of 1 cm^3 substrate per 0.2 cm^3 of Ca-alginate beads entrapped with CPO was incubated at 20°C with constant shaking. 1.0 cm aliquots of supernatant were taken periodically in each 5 min, placed in a boiling water bath for additional 30 min and used to determine optical absorbency. The greatest change in absorbency per min was applied for further work.

Phosphatase activity determination.

The standard analysis for phosphatase activity determination is p-nitrophenylphosphate (pNPP) hydrolysis (Torriani, 1960). After the enzyme incubation (50 μ M) with pNPP (15 mM, 400 μ l) the reaction was stopped with adding of 1 ml 0.5 M NaOH and the amount of pNP was determined spectrophotometrically at 410 nm (ϵ (pNP) = 17,9 mM⁻¹cm⁻¹ at pH 12).

When determining activity of immobilized CPO in Ca-alginate beads 20 beads (0.1g) (the amount of CPO in immobilized preparations was the same as for control measurements) were placed into a batch and 400 μ l of 15 mM pNPP was added. The reaction mixture was incubated for 60 min at 37°C. The aliquotes of the supernatant were taken away and the reaction was stopped with adding of 1 ml of 0.5 M NaOH. Then they were used to determine amount of pNP.

Determination of protein concentration

The concentration of the immobilized protein in the beads containing BSA was determined:

- 1) from the difference between the amount of protein (CPO) introduced to the coupling reaction mixture and the amount of protein present in the filtrate and washings after immobilization;
- 2) (for Ca-alginate beads only) by destroying the beads with the stirrer (type ML-4, Poland) in 0.1M NaAc buffer at pH 5.5. The suspension was then centrifuged at 2000 rpm and the supernatant assayed for protein content.

The amount of protein was determined spectrophotometrically at 280 and 260 nm using the following equation: protein (mg ml⁻¹) = 1.55 A₂₈₀ – 0.76 A₂₆₀ and by the Lowry method.

Stability test

Activity of immobilized CPO for the first cycle was determined as described above. When 70% bromphenol blue liberated, the remaining supernatant fluid was decanted and the beads were thoroughly washed with 0.01M NaAc containing 10 mM CaCl₂ and assayed for activity for the second cycle and so forth.

Results and Discussion

Entrapment of CPO in calcium-alginate beads

Attempts were made to immobilize CPO by entrapment in uncoated calcium alginate beads and to stabilize them by coating with hardening reagents such as GA and PEI or entrapping the enzyme together with stabilizing agent potassium ferricyanide.

A preliminary experiment was made to determine the appropriate concentrations of hardening and stabilizing agents. Hardening reagents usually have an appreciable detrimental effect on the free enzyme (Woodward, 1988; Lee and Siaw, 1993). CPO is not an exception - its residual activity is not greater than 10% when treated the free enzyme whereas a loss of enzyme activity in immobilized preparations under the treatment with hardening reagents was found to be not significant (not shown). However, when the beads were subjected to the 2nd cycle of reaction and so forth, it was appeared that there were no changes in operational stability under the concentrations of hardening reagents greater than 0.5%. Therefore, the concentration of 0.5 was used for further work. It was estimated that the process used in the case of hardening reagents resulted in about 30% of the enzyme being

entrapped in the beads and for beads with the stabilizing agent in about 25%.

Specific activity and kinetic constants estimation

Specific activities and kinetic constants of the free and immobilized forms of the CPO are summarized in Table 1.

In general the specific activities of immobilized in calcium alginate beads enzyme are higher than that of the free CPO. This is probably due to the higher local concentration of substrates in the immobilized systems. The beads coated with hardening reagents showed a decrease in activity especially in the case of PEI in comparison with unmodified beads but it was not so significant as for the free enzyme as emphasized earlier. PEI in the presence of Ca²⁺ can reversibly form gel that causes more hydrophobic environment for the enzyme (Joung, 1987). The last can possibly decrease the activity of CPO. However, the adding of stabilizing substance such as potassium ferricyanide caused an increase in activity including the variants of beads which were coated with hardening agents. In the case of ferricyanide it is possible that stable complex ferricyanide anion can form some additional coordinating bonds and change the conformation of the active center of the CPO.

Although the beads containing potassium ferricyanide and BSA were more stable when stored as described above (data not shown) the adding of BSA always caused a decrease in activity of the immobilized CPO (data not shown). In particular, it can be seen from the difference in activity between the beads containing potassium ferricyanide and the beads containing potassium ferricyanide and BSA. Therefore, these beads were not characterized further.

To determine the Michaelis constants (K_M) and maximal velocities (V_{max}) the effect of Br⁻ concentration on the activity of CPO was investigated. The K_M and V_{max} values were estimated from Lineweaver-Burk plots. The highest K_M values for beads coated with hardening reagents can be explained through the resulting tighter and smaller pores of the beads conferring greater resistance for the diffusion of the substrate (Koilkpillai, 1990). The V_{max} values for coated beads were the lowest in according with the K_M . However, the K_M values of immobilized systems were found to be lower and V_{max} , respectively, higher than that of the free enzyme. This effect was observed probably due to the influence of the matrix. Through hydrophobic interaction of the carbohydrate chain of alginate molecule with the aromatic substrate, the alginate matrix provides sites for concentration of the substrates on the beads and thus creates higher local concentration of substrates in closer proximity to the enzyme molecules (Woodward, 1986).

Operational and thermal stability

A number of experiments in batch system were set up to investigate the operational stability of the immobilized enzyme.

Fig. 1A shows the plot of percentage relative activity of various immobilized systems against the number of reaction cycles, where the activity for the first reaction cycle was assigned a value of 100%. We can find a great decrease of CPO activity for the uncoated beads after the first reaction cycle and only 5% residual activity was detected after 5 reaction cycles. This is may be due to the leakage of the enzyme from the beads what is the most serious problem when immobilizing enzymes in calcium alginate gel (Nahalka et al, 2004). The possibility of inhibition with the product of reaction - bromphenol blue - is also not excepting.

Fig. 1B shows the activity of immobilized CPO in various types of calcium alginate beads after each reaction cycle. Although the same amount of beads containing approximately the same quantity of the enzyme were used in each immobilized system, PEI-coated calcium alginate beads exhibited lower activity as compared to other systems for all reaction cycles. It can be explained with the influence of polycationic reagent PEI as far as it states that the charge of a matrix may have great importance in the case of redox reaction (Coilpillai, 1990). However, the adding of potassium ferricyanide increases the activity of immobilized enzyme markedly both uncoated beads and beads coated with hardening reagents. In result, the activity of coated with glutaraldehyde was almost the same as uncoated beads. The activity of immobilized CPO in PEI-coated beads decreases slightly after the first and for the subsequent reaction cycles. The beads coated with GA showed 40% residual activity after the first reaction cycle and this figure increased to 70% for the beads containing potassium ferricyanide. This indicates that the calcium alginate beads were stabilized after being treated with hardening reagents such as PEI and glutaraldehyde and $K_3[Fe(CN)_6]$ stabilized the enzyme being included into beads.

To investigate the thermal stability residual activities of free and immobilized enzyme were determined with the standard method after the incubating the enzyme samples at temperatures in the range 20 - 85°C for 10 min. Above 45°C the stability of all the systems began to decrease markedly (not shown).

The activity of 100% refers to the rate of halogenation at 45°C by each of the systems studied. In the whole, the thermal stability of the free enzyme was higher than that of the immobilized CPO. Therefore the adding of potassium ferricyanide seems very useful, as it raises the thermostability of the immobilized enzyme up to the level of that of free CPO possibly due to new coordinative bonds formation. The most thermostable were the beads containing potassium ferricyanide and glutaraldehyde – coated. They lost only 38% of their activity after the treatment at 65°C in contrary to unmodified beads losing 52.5%.

Optimum temperature and pH

The results obtained for the optimum temperature of both the free and immobilized CPO are shown in Fig. 2b. The most effective process takes place at 45°C for all the systems. The optimum temperatures for the reaction were in the range of temperatures at which the immobilized enzyme was the most thermostable. This renders the process more efficient.

The effect of pH on the bromination of phenol red was also investigated. The buffer used in this study was 1 mol dm⁻³ NaAc containing 10 mmol dm⁻³ CaCl₂ (pH 4-7). The optimum pH of the enzyme was found to be almost the same for all immobilized forms (Fig. 3). The activity of free and immobilized CPO was constant at pH between 5.2 – 6.0. Only in the case of PEI- coated beads pH optimum shifted slightly to lower pH values probably due to the effect of polycationic hardening reagent (Tanaka et al., 1984).

Halogenating activity determination of CPO immobilized in semi-permeable membranes

We optimized conditions for investigation of halogenating activity of immobilized CPO in semi-permeable membranes. The next

parameters were varied: concentration of a carrier protein, cross-linking agent concentration and the time of treatment with a cross-linking agent (These preliminary data are not shown). The last parameter was varied only for membranes containing gelatin due to different preparation technique. When investigating the effect of BSA and GA concentration on the activity of preparations containing BSA it has been found that the preparation with 1.35% BSA and 0.9% GA possesses the highest halogenating activity. If to increase the concentration of BSA and/or GA the activity of immobilized CPO reduces. When using gelatin as a protein carrier the preparation treated with 0.5 % GA for 2 min has the highest halogenating activity. In the further investigations the pointed concentrations were used. In the case of RNAase or Cyt C as a protein carrier the concentration of 3 % is the greatest for halogenating activity determination. However, the activity and/or stability of immobilized with RNAase or Cyt C CPO is much more lower than with the other protein carriers so they are not used in the further investigations. The data on immobilization conditions and activity of CPO immobilized in different types of semi-permeable membranes are summarized in Table 2.

Operational stability of immobilized CPO preparations (halogenating activity)

Operational stability is the best for the membranes with BSA (Preobrazhenskaya et al, 2006). It can be seen easily from first 5 recycles. However, after the second recycle the activity rises to more than 100 %. It can mean that the membrane destroys under reaction conditions. Moreover, to the disadvantages of this preparation low residual activity of 3.5 % can be adhered. In the case of gelatin used as a protein carrier the most stable immobilized CPO is a preparation with 3% gelatin, 2.5% GA and the time of GA treatment 3 min. (Fig. 4A). The activity remains rather high during 7 recycles then it begins to decrease. The most useful GA concentration using gelatin as a protein carrier is 2.5%. As it can be seen from Fig. 2 there is a slight dependence on the time of GA treatment.

As seen from our previous data and Fig. 4B, illustrating operational stability obtained for preparations of immobilized CPO the best ones are with gelatin as a protein carrier. They possess high operational activity and their operational stability is not less than 65% during 7 recycles. The preparations of immobilized CPO with gelatin are the most perspective for further usage.

Phosphatase activity of free and immobilized CPO

Effect of substrate concentration on reaction rate. It is stated that at concentrations of pNPP from 6 mM till 15 mM the dependence of reaction rate on the substrate concentration is linear (not shown). For further measurements the concentration of pNPP of 15 mM was used.

Specific phosphatase activity and reaction velocity of immobilized CPO preparations. In Table 3 there are results on phosphatase activity of the immobilized CPO. Relative, specific activity and reaction velocity are presented. The relative activity of CPO was determined in comparison to the activity of free enzyme which is valued as 100%. As it can be seen the membrane made of BSA with Cyt C (1.15 % from total protein) possesses the least relative phosphatase activity. It is probably because although BSA stabilizes many proteins and the CPO during storage too but the

activity of the enzyme in the presence of BSA reduces (unpublished data).

Operational stability and stability under storage of immobilized CPO during phosphatase activity determination. CPO immobilized in gelatin membranes with 0.75% GA possesses the best operational stability among the systems presented when phosphatase activity being determined (Fig.4B). The time of GA treatment for preparations of CPO with gelatin was 3 min. One can observe the increasing of phosphatase activity of CPO higher than 100% in the membranes with BSA after the second recycle. It may say about partial degradation of the membrane under the reaction conditions and the pNPP hydrolysis becomes easier for the free enzyme. It can be confirmed with the similar data measuring halogenating activity of the same immobilized CPO preparations (Preobrazhenskaya et al, 2006).

The operational stability of calcium alginate beads under phosphatase activity determination is rather low that can be connected with gel degradation due to presence of phosphate ions. As it is known from the literature a phosphate ion destroys calcium alginate gel (Woodward, 1986).

We investigated the stability of CPO immobilized in the membranes on the base of gelatin and GA- coated in comparison with Ca-alginate beads containing the enzyme in phosphate buffer as interior medium. The Phosphatase activity of CPO was determined in a day. The stability of the membranes with BSA and especially with gelatin conserves high during 10 days – the activity dropped only for 20 ± 3 % nM per minute for 1 mg of protein (the data not shown). The enzyme preparation immobilized in Ca-alginate beads has significantly lower phosphatase activity after ten days of storage – 55%. This is different from the expected result taking into consideration that the phosphatase activity of CPO after purification in phosphate buffer increases twice due to avoiding competition with acetate ions in the active center (Preabrazhenskaya et al, 2003). The decreasing in stability can be explained with degradation of the beads during storage in 0.1M phosphate buffer, pH5.5. As it was said above the alginate gel can be destroyed in the presence of phosphate ions (Woodward, 1988). The defense of the active center of the enzyme with phosphate ions in the case of enzyme purification as described in [7] does not play any significant role in the gel.

Substrate specificity of CPO when determining phosphatase activity

Substrate specificity of CPO is expressed in the figures of specific activity. As 100% the specific phosphatase activity of creatinephosphate (CP) was assigned (Table 4). Phosphatase activity in this assay was determined on increasing of inorganic phosphate concentration when the concentration of substrate was 1 mM. The next law was found when using di- and triphosphates as substrates: on CTP and ITP the specific activity is twice higher and on UTP and GTP 1.5 times higher than on monophosphates except creatinephosphate (CP). When determining phosphatase activity on pNPP the dependence on the presence of inorganic phosphate is showed: the phosphatase activity of CPO increases more than twice in 5 mM K- phosphate buffer (pH 7.5).

We accounted the concentration of a separate hydrolyzed substrate during the reaction. It was necessary to confirm that measurements were carried out at the steady state of the reaction. For calculations the next equation based on Bouguer-Lambert-Bear law was used:

$W = \Delta Dv/\epsilon l t$ [25], where:

W – the reaction rate, mmol min⁻¹ mg⁻¹;

ΔD – the absorbency change per min;

v – sample volume, ml;

ϵ – molar extinction coefficient equal to $18\,300\text{ M}^{-1}\text{cm}^{-1}$;

l – the density of a light-absorbing layer of a cuvette.

In Table 4 the concentration of hydrolyzed substrate is expressed in percentage from the initial concentration. For all the substrates it was showed that the phosphatase activity of CPO has been determined at the beginning of the reaction. 0.26 % to 1.23 % of the substrate used is hydrolyzed for 60 min. Interestingly, the bacterial haloperoxidase possessing phosphatase activity can hydrolyze creatinephosphate – a source of energy in mammalian muscles! According to the data on the substrate specificity of CPO we can state the CPO possesses rather wide substrate specificity and can be successfully used in many technological processes.

Estimation of the obtained preparations of CPO in a column bioreactor

To estimate the immobilized CPO preparations in use a fluidized-bed or fixed-bed (for calcium-alginate beads only) column bioreactor 5×0.5 cm was applied. Ca-alginate beads were spread at the column, they did not flow up as it often occurs in fluidized-bed bioreactors (Gilson and Thomas, 1995). Semi-permeable membranes were placed as a volume spiral into the column. The column with CPO immobilized in beads has worked for 5 days with decreasing of 53 % in halogenating activity of CPO when using MCD as a substrate. With phenol red as a substrate the activity significantly dropped to 25% in two days however it stayed stable at that level for quite a long time (Fig. 5A).

For semi-permeable membranes containing immobilized CPO and gelatin the yield of product was almost the same when MCD or phenol red used as a substrate (Fig 5A). Moreover, it is high – about 80 % and quite stable for 10 days of the reactor running. As it states, the apparent K_m for substrate in semi-permeable membranes depends on diffusion coefficient, thickness of membrane and concentration of enzyme in a membrane (Kolisis, 1987). Probably, with fluidized flow of substrate K_m can be decreased due to less diffusion limitations as far as external diffusion is a function of linear flow rate (Laskin, 1987). It can also be confirmed by (Berezin et al, 1976) where they state that diffusion can be decreased if to make substrate flow through the column. In the opposite, if the rate of the reaction depends on the rate of substrate flow it indicates the existence of diffusion limitations of the process (Berezin et al, 1976). To confirm this statement the K_m value for the enzyme entrapped in gelatin membrane treated with 0.5 % GA was determined. It is 92 ± 7 mM of Br⁻ when using MCD as a substrate, K_m for free enzyme is 69 mM. The other possibility is that if diffusion limitations in continuously stirred type of a bioreactor are less for enzymes inhibited by substrate then fully back-mixing reactors are the least efficient for enzymes with product inhibition (Laskin, 1986). Thus, for calcium-alginate beads under measuring phosphatase activity with the substrate pNPP the yield drops till 56% after 2 days that was registered when determining the effect of flow rate on the yield of product in the column bioreactor (Fig.5B) although in a fixed-bed column the yield drops to 56% after 6 days of function (Fig 5A).

It is interesting to observe that the yield of product for phenol red under determining recyclability in a batch system is much higher in comparison to that in a fluidized bioreactor. Probably it can be explained with product inhibition since it has been mentioned already that the reaction product tetrabromophenol blue inhibits CPO. This type of a reactor is the least efficient in such a case (A. Laskin, 1986). For pNPP this observation is not due: the yield of product (pNP) is much higher when using a fluidized column bioreactor. As for MCD we can only state that for gelatin membranes the situation is the same: the yield is higher in the fluidized bioreactor. Probably it is concerned to the size of a substrate molecule: a molecule of phenol red is approx. 3 times larger than MCD and pNPP, it has 3 phenol rings instead of 1 in another 2 substrates and probably gives sterical obstacles flowing through the column. But when the reactor has semi-permeable membrane rolled as a spiral and directed along the column, in the same direction as substrate flow, instead of Ca-alginate beads sterical limitations are not so obvious. That is for gelatin membranes there is almost no difference when using MCD or phenol red. It can be confirmed determining the dependence of product yield on the substrate flow through the column.

As it can be seen from Fig. 5B, the yield of the product depends on the substrate flow rate through the column. The yield in the reactor occurred exceptionally high when using MCD as a substrate – 91 %. It can say about the perspective of usage of such a method in production. The yield of bromophenol blue is less – 63 % and for pNP under measuring phosphatase activity – 56 %. In average the rates between 25-35 ml hour⁻¹ are applicable. For MCD the highest yield was obtained under flow rate of 27 ml hour⁻¹, for phenol red and pNPP – 32 ml hour⁻¹.

Conclusion

Non-heme CPO from *Serratia marcescens* was successfully immobilized in calcium alginate beads and semi-permeable protein membranes. These systems may provide a suitable matrix as an efficient, economical and rapid method for immobilization of bacterial non-heme haloperoxidases. In a fluidized-bed bioreactor one can continuously obtain halogenating products using gelatin membranes with the immobilized CPO, when using Ca-alginate beads coated with GA and containing ferricyanide continuous hydrolysis of organic phosphates is possible.

References

1. Neidleman, S.L., Geigert J. 1986. Biohalogenation: principles, basic roles and applications. Chicester, UK: Ellis Horwood, pp.46-61.
2. van Pee K.-H., 1996. Biosynthesis of halometabolites by bacteria. Annu. Rev. Microbiol. 50:375-399.
3. Pelletier, I., Pfeifer, O., Altenbuchner, J., van Pee, K.-H. 1992. Molecular cloning and sequencing of a non-haem bromoperoxidase gene from *Streptomyces aureofaciens* ATCC 10762. J. Gen. Microbiol. 138: 1123-1131
4. Pelletier, I., Pfeifer, O., Altenbuchner, J., van Pee, K.-H. 1994. Cloning of a second nonhaem bromoperoxidase gene from *Streptomyces aureofaciens* ATCC 10762: sequence analysis, expression in *Streptomyces lividans* and enzyme purification. Microbiol. 140: 509-516.

5. Preobrazhenskaya, Yu.V., Voskoboev, A.I., Burd, V.N. 2003. Hydrolysis of Phosphoester bond by the Heme-Independent Chloroperoxidase from *Serratia Marcescens*. Russian J. of Bioorg. Chemistry.- V.29. No.6. P.556-559.
6. Burd, W., Yourkevich, O., Voskoboev, A., van Pee, K.-H. (1995). Purification and properties of a nonheme chloroperoxidase from *Serratia marcescens*. *FEMS Microbiol. Lett.* **129**. 255–260.
7. Preobrazhenskaya, Yu.V., Voskoboev, A.I., Burd, V.N. 2003. Phosphatase activity of non-heme chloroperoxidase from the bacterium *Serratia marcescens*. *FEBS Lett.* 536: 41-44.
8. Nahalka J, Wu B, Shao J, Gemeiner P, Wang PG. 2004. Production of cytidine 5'-monophospho-N-acetyl-β-D-neuraminic acid (CMP-sialic acid) using enzymes entrapped in calcium pectate-silica-gel beads. *Biotech Appl. Biochem.* 40: 101-106.
9. Lee KH, Lee PM, Siaw YS. 1992. Studies of phenylalanine production by immobilized aminoacylase. *J. Chem. Tech. Biotechnol.* 54: 375-382.
10. Berezin, I., Antonov, V., Martinek, K.(Ed.) (1976). Immobilized enzymes. V.2. MGU, Moscow. - 458p.
11. Gilson, C.D., Thomas, A. (1995) Ethanol production by alginate immobilized yeast in a fluidized bed bioreactor. *J. Chem. Tech. Biotechnol.* **62**. 38-45.
12. Joung J.J., Akin C., Roger G.P. (1987) Immobilization of growing cells by polyethylenimine in modified alginate. *Appl. Biochem. Biotechnol.* **14**. 259–275.
13. Koilpillai L. (1990) Immobilization of penicillin G acylase on methacrylate polymers. *J. Chem. Tech. Biotechnol.* **49**. No.2. 173-178.
14. Kollis, F.N., Thomas, D. (1987) Continuous Assay of Neuraminidase Activity by a Bienzyme System immobilized in an Artificial Membrane. *Biotech. and Bioeng.* **30**. 160–163.
15. Laskin A. (Ed.) 1986. Enzymes and Immobilized Cells in Biotechnology. London, UK. 341p.
16. Lee KH, Lee PM, Siaw YS. 1992. Studies of phenylalanine production by immobilized aminoacylase. *J. Chem. Tech. Biotechnol.* 54: 375-382.
17. Lee K.H., Siaw S.Y. 1993. Covalent immobilization of aminoacylase to alginate for L- Phenylalanin production. *J Chem Tech Biotechnol.* 58: 65-70.
18. Morris DR, Hager LP. 1966. Chloroperoxidase 1. Isolation and properties of the crystallin glycoprotein. *J. Biol. Chem.* 241: 1763-1768.
19. Preobrazhenskaya Y., Burd V., Voskoboev A. 2000. Phenol red – a substrate for determination of activity of immobilized preparations of haloperoxidases. *Vestnik GrGU. Belarus, №1. Ser.2*, 92-97.
20. Torriani A.M. 1960. *Biochim. Biophys. Acta.* 38:460-469.

21. Woodward, J. 1988. Immobilized Cells and Enzymes. Methods. Moscow: Mir, 215p.
22. Gerard E.F., Thirakorn Mekkawes, Linus O. Johannissen, Warwicker J., Spiess, Reynard R., Blanford Christopher F., Hay Sam, Heyes Derren J., and de Visser Sam P. *How Is Substrate Halogenation Triggered by the Vanadium Haloperoxidase from Curvularia inaequalis?* ACS Catalysis 2023 13 (12), 8247-8261.
23. Preobrazhenskaya Y., Bogdevich Yu., Burd V. Immobilization of Chloroperoxidase from *Serratia marcescens* in semi-permeable protein membranes. Appl. Biochem. Microbiol. 2006, 42, No. 2. P.134-137.PMID: 11530668.
24. Jennifer Littlechild, Esther Garcia Rodriguez, Mikhail Isupov. Vanadium containing bromoperoxidase--insights into the enzymatic mechanism using X-ray crystallography. J. Inorg.Biochem. 2009. Apr. 103(4): 617-21.
25. Severin,S.E. Solovieva GA. 1986. Handbook on Biochemistry. Moscow, MSU edition. -in Russian, 509p.

TABLE 1.

Specific activities and kinetic constants of free and immobilized CPO.

Sample	Activity* $\mu\text{mol min}^{-1}$	Protein, $\mu\text{g ml}^{-1}$	K_M , mmol ml^{-1}	$V_{\max}$, mmol min^{-1}	Specific activity*, $\mu\text{mol min}^{-1}\text{mg}^{-1}$
Free CPO	3.30	40.0	210	32.0	85.0
Beads:					
1) unmodified	0.42	3.8	180	34.6	110.5
2) with $\text{K}_3[\text{Fe}(\text{CN})_6]$	0.56	3.9	156	42.4	142.5
3) $\text{K}_3[\text{Fe}(\text{CN})_6]$ and BSA-containing	0.41	3.8	178	34.8	106
4) GA-coated	0.33	5.3	422	21.5	62.1
5) PEI-coated	0.09	4.7	476	9.2	18.8
6) with $\text{K}_3[\text{Fe}(\text{CN})_6]$ and GA-coated	0.42	4.0	255	32.9	105
7) with $\text{K}_3[\text{Fe}(\text{CN})_6]$ and PEI-coated	0.25	3.9	302	20.8	64.6

* - haloperoxidase activity of free and immobilized CPO was measured using phenol red as a substrate.

TABLE 2.

Immobilization conditions and activity of CPO immobilized in different types of semi-permeable membranes.

Protein carrier, concentration, %	GA concentration, %	Residual activity, %	Activity for 1 preparation, U
BSA, 1.35 %	0.9	4.3 ± 0.3	0.34 ± 0.03
Gelatin, 3 %	0.5	10.5 ± 0.4	0.82 ± 0.03
RNAase, 3 %	0	2.9 ± 0.3	0.23 ± 0.02
Cyt C, 3 %	0	4.4 ± 0.3	0.34 ± 0.02

TABLE 3.**Specific phosphatase activity and reaction velocity for immobilized CPO preparations (substrate- pNPP).**

Immobilized CPO preparation	Relative phosphatase activity, %	Specific activity, $\text{nmol min}^{-1} \text{mg}^{-1}$ of protein	V, nmol min^{-1}
Membranes with BSA	23.4	36 ± 3	$8.71 \pm 0,7$
Membranes with gelatin	16.9	19 ± 2	$3.93 \pm 0,3$
Ca –alginate beads:			
with Na-acetate buffer	54.7	81 ± 7	$19.68 \pm 2,0$
with Tris buffer	39.1	58 ± 5	$14.21 \pm 1,2$
With K-phosphate buffer	26.5	40 ± 4	$12.11 \pm 1,0$
Membranes with gelatin and 0.5% GA	48.3	69 ± 6	$16.87 \pm 0,6$
Membranes with gelatin and 0.75% GA	27.9	39 ± 3	$9.55 \pm 0,8$
Membranes with BSA+Cyt C	14.4	22 ± 2	$5.20 \pm 0,5$

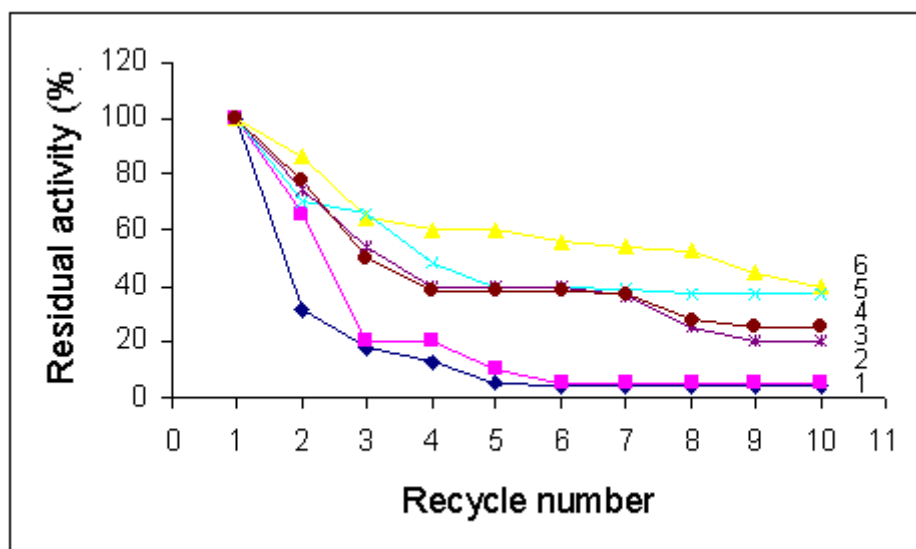
TABLE 4.**Reaction velocity and concentration of hydrolyzed substrate (phosphatase activity of immobilized CPO)**

S	Relative phosphatase activity, %	W, $\mu\text{mol min}^{-1} \text{mg}^{-1}$ of protein	[S], %
CP	100	12.306 ± 0.011	1.23 ± 0.011
pNPP in 5 mM KPP buffer	64	7.715 ± 0.06	0.77 ± 0.0068
CTP	50	6.185 ± 0.056	0.618 ± 0.0055
UTP	40	6.153 ± 0.055	0.61 ± 0.053
GTP	40	5.02 ± 0.045	0.50 ± 0.0045
IDP	50	4.98 ± 0.043	0.49 ± 0.044
pNPP	25	3.355 ± 0.03	0.33 ± 0.0028
GP	27	3.278 ± 0.028	0.32 ± 0.0027
G-6-P	21	2.622 ± 0.024	0.26 ± 0.0024

Note. CP – creatinephosphate, GP – glycerolphosphate

FIGURES

A



B

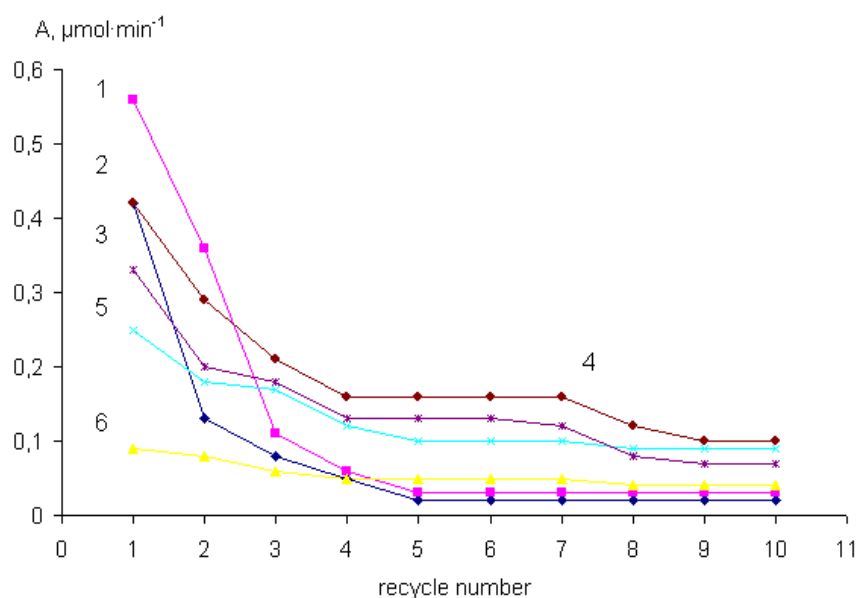


FIG. 1. Operational stability of CPO immobilized in various forms of Ca-alginate beads. (A) Plot of % relative activity against the reaction cycle number for various immobilized systems. (B) Plot of activity against the reaction cycle number for various immobilized systems. Ca-alginate beads: 1- uncoated; 2 - K₃[Fe(CN)₆]-containing; 3 - GA-coated; 4 - K₃[Fe(CN)₆]- containing and GA-coated; 5 - PEI-coated; 6 - K₃[Fe(CN)₆]-containing and PEI-coated.

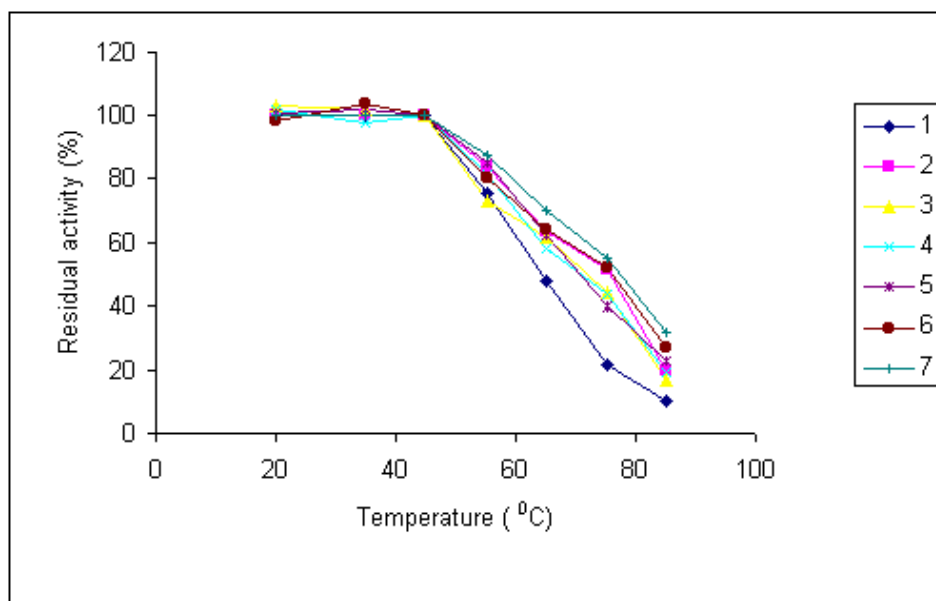
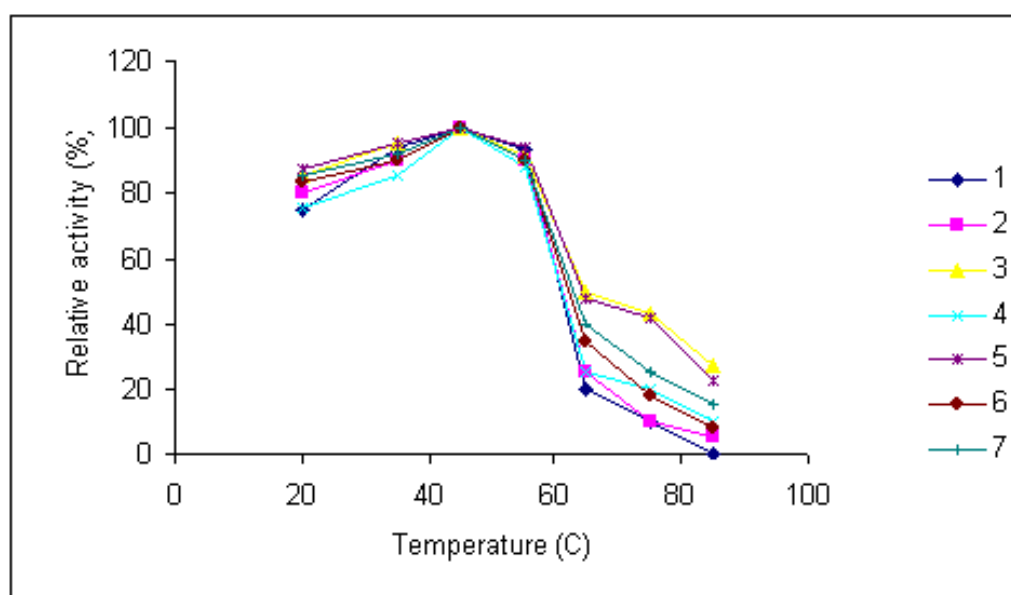
A**B**

FIG. 2. Effect of temperature on the (A) stability of CPO. A mixture of 0.6 cm^3 of $0.5 \text{ mmol dm}^{-3} \text{ CaCl}_2$ and 0.1 g of Ca-alginate beads entrapped with enzyme were incubated at various temperatures for 10 min. It was then rapidly cooled in an ice bath. The residual activity was then assayed by the standard method. The determined residual activities at various temperatures were calculated with respect to that at 45°C . and (B) on the activity of CPO. The activities at various temperatures were measured by the standard method. They were then calculated with respect to that at 45°C . 1 – uncoated beads; 2 - free enzyme; 3 – beads with $\text{K}_3[\text{Fe}(\text{CN})_6]$; 4 – GA-coated beads; 5 – beads with $\text{K}_3[\text{Fe}(\text{CN})_6]$ and GA-coated ; 6 – PEI-coated beads; 7 - beads with $\text{K}_3[\text{Fe}(\text{CN})_6]$ and PEI-coated.

Optimum pH for free and immobilized CPO

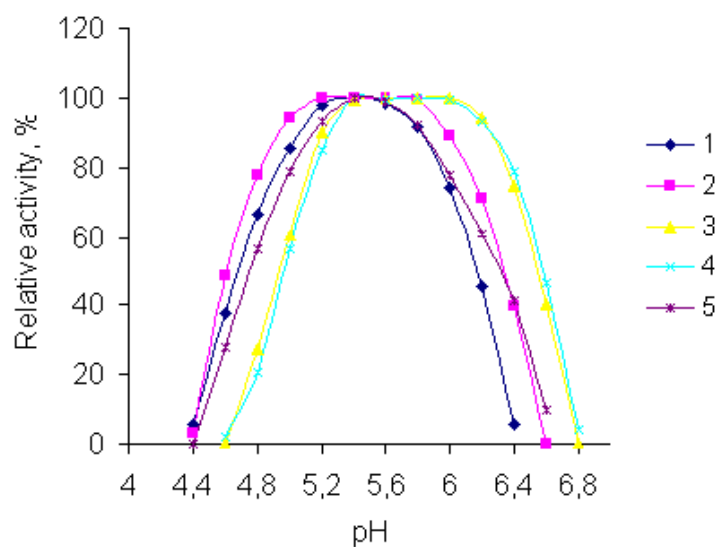


Fig. 3. Optimum pH for free and the immobilized in beads CPO (substrate-phenol red). 1- free enzyme, 2- PEI-coated beads; 3- unmodified beads, 4- GA-coated beads, 5- beads with $K_3[Fe(CN)_6]$.

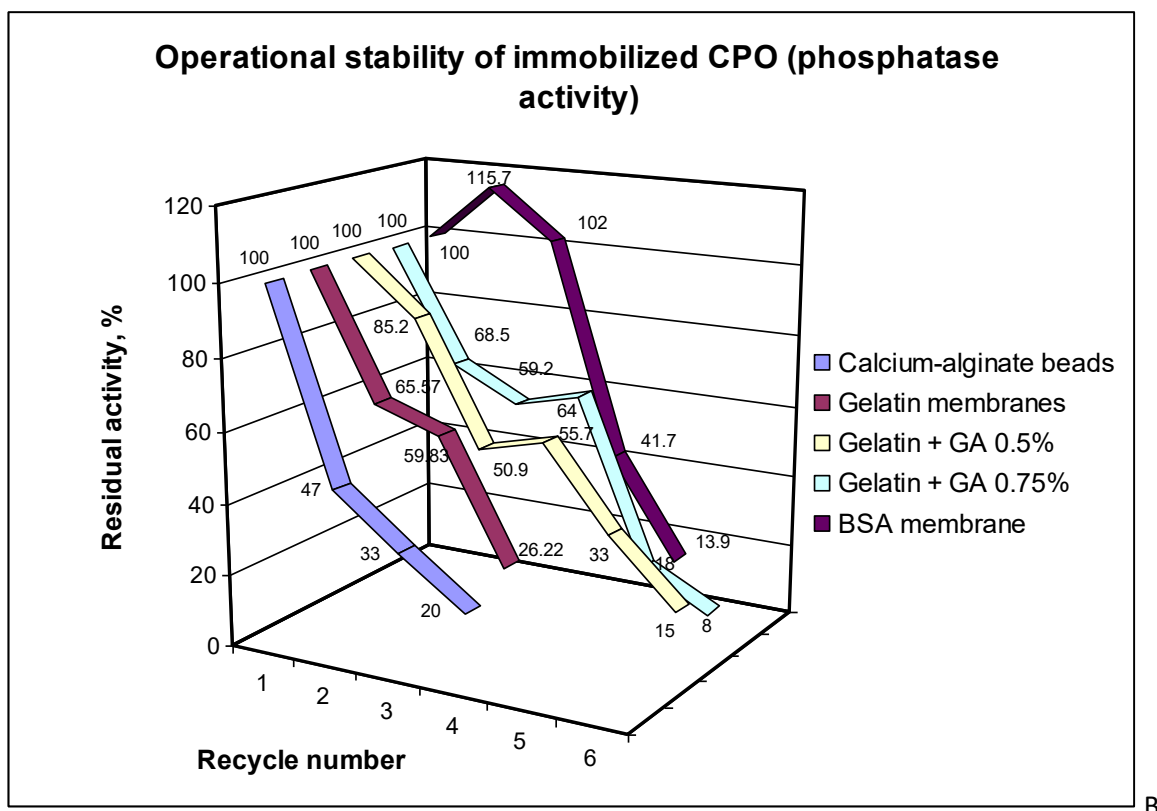


FIG. 4. Operational stability of immobilized CPO under determining haloperoxidase activity in semi-permeable membranes (A) and under determining phosphatase activity (B). Plot of % relative phosphatase activity against the reaction cycle number for various immobilized CPO systems.

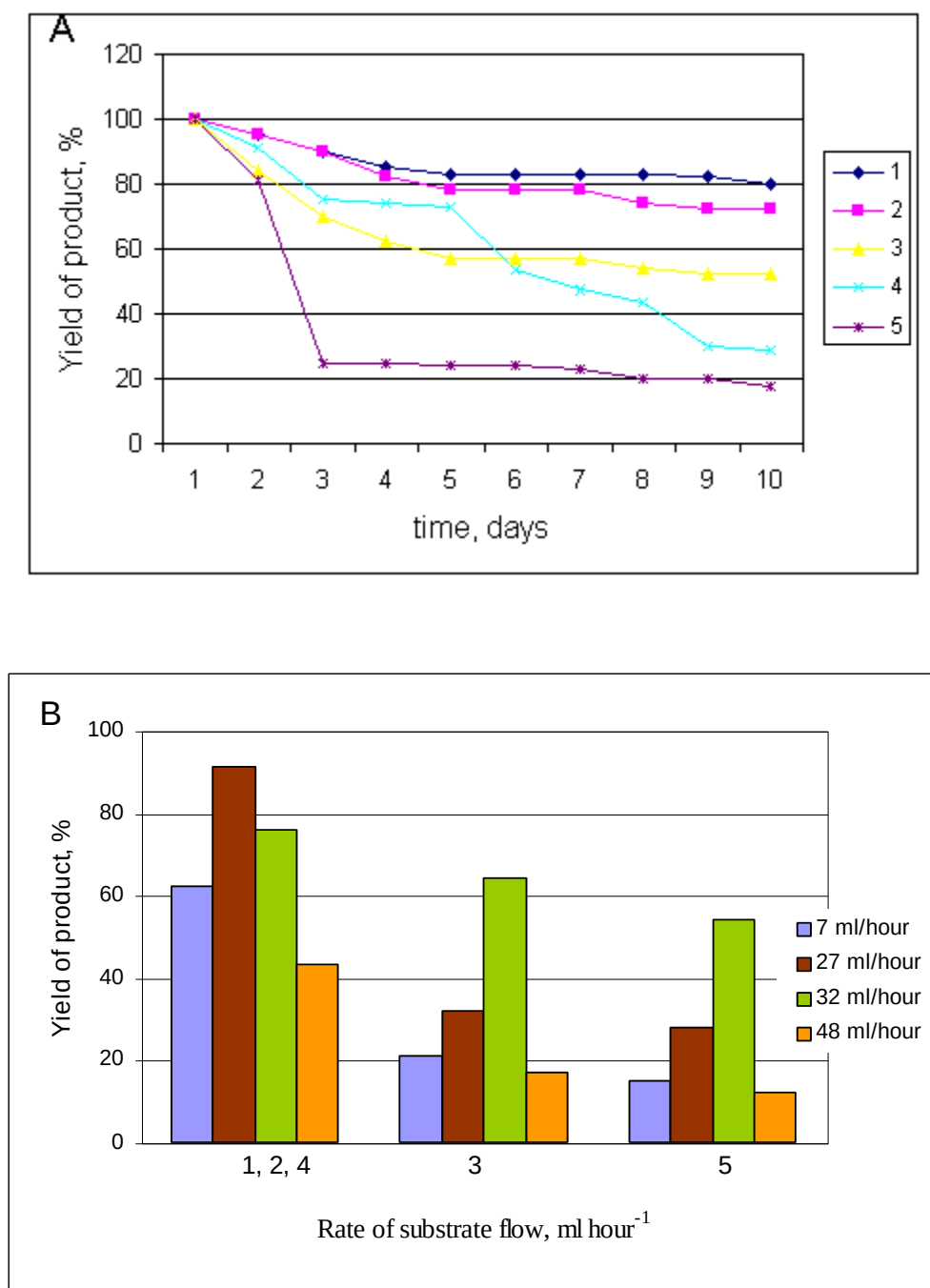


FIG 5. Yield of products in the bioreactor in dependence on time (A) and on rate of substrate flow (B). 1- membranes on the base of gelatin, 0.5 % GA, substrate – MCD; 2 - membranes on the base of gelatin, 0.5 % GA, substrate – phenol red; 3 – Ca –alginate beads, substrate – pNPP; 4 - Ca –alginate beads, substrate – MCD; 5 - Ca –alginate beads, substrate – phenol red.