04-3	**A novel method of soil bioremediation with immobilized enzyme preparation**	

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INTRODUCTION AND OBJECTIVES

Organophosphorous compounds (OPC) that are derivatives of orthophosphoric acid and alkylphosphonic acid find widespread use in agriculture as pesticides [Nollet 2011].

The yearly large-scale use of OPC [Soumis 2000], as well as low decomposition rates of OPC in the environment cause the accumulation of these compounds in soils, from which they are subsequently washed out to enter groundwater and rivers. [Riley 2000].

The toxic effect of OPC has a cumulative nature and entails various degenerative disorders of the nervous system [Kwong 2002]. Due to this, efficient and environmentally friendly techniques for eliminating OPC from soils are of major practical interest.

From the environmental and economic standpoint, the most practical method is the decomposition of OPC in soils *in situ* using biocatalysts, which can include enzymes capable of destructing toxic OPC. The application of the enzyme in the immobilized form allows to increase its stability in soil, as well as to increase its persistence in the place of introduction into the soil.

Organophosphorous hydrolase (OPH, EC 3.1.8.1), containing six residues of histidine on N-terminus of protein molecule (His_6-OPH), hydrolyses organophosphorus pesticides and warfare agents (Efremenko 2001). That provides expediency of using this enzyme for OPC destruction and soil bioremediation.

MATERIALS AND METHODS

Wheat sawdust was used as carrier for immobilization. Paraoxon, Parathion, Methylparathion and Diazinon were obtained from Sigma. Soil samples were provided by the Soil Science Department of the M.V. Lomonosov Moscow State University. Ethyl acetate AR, hexane AR and acetonitrile AR were obtained from Reachim (Moscow, Russia).

His_6-OPH was used as a non-purified preparation, obtained after desintegration by sonifier of *E.coli* SG13009[pREP4] cells (Efremenko 2005)

In order to obtain the immobilized enzyme preparation, 300 ml of the unpurified His_6-OPH (200 U ml^{-1}) in 0.1 M phosphate buffer (pH 7.4) were mixed with 100 g of the carrier and left at 8°C for 20 min with periodic stirring (Efremenko 2011).

In order to determine the concentrations of Paraoxon (PX), Parathion (PT), the pesticides were extracted from 3 g of soil with three portions of ethyl acetate (5 ml). The extracts were mixed, evaporated and dissolved in acetonitrile. Pesticide extracts were analyzed using HPLC (Knauer Smartline Pump 1000, Germany) and a Diasfer 110-C18 reverse-phase chromatography column (Biochemmack CT) (5 μm, 4.0 × 250 mm) with a spectrophotometric detector (λ = 274 nm) and isocratic elution (CH_3CN:H_2O = 60:40). The retention time for PX, and for PT was 4.5 min, 20 min, respectively. The eluent flow rate was 1 ml min-1. The sample volume was 20 μl. The same procedure was done for Diazinon with some exceptions (extragent – acetone, λ = 225 nm, retention time – 19 min)

In order to carry out bioremediation experiments, immobilized His_6-OPH was introduced into flasks with contaminated soils (1 g_{straw} $kg^{-1}_{dry\ soil}$).

RESULTS AND DISCUSSION

The screening of different carriers for the immobilization of His_6-OPH showed that the maximum of enzymatic activity was established for straw as carrier (72 U g^{-1}, well-washed carrier). Literary analysis shows that wastes sawdust and agriculture can act not only as ecologically acceptable and cheap carriers for enzymatic immobilization, but also as soil structurators (Mahmoud 2007).

During the investigation straw's maximal water-absorbing capacity was established as 2.5 ml. Variation of supernatant activity showed that the optimal activity is 200 U ml^{-1}. On the basis of obtained results optimal parameters for His_6-OPH immobilization on the straw were defined.

The enzymatic hydrolysis efficiency of various organophosphorous pesticides under the action of the enzyme immobilized on wheat straw in various soils samples has been studied; the characteristics of the latter are presented in Table 1.

Studies of OPC hydrolysis under the action of the immobilized enzyme (300 U kg^{-1}_{soil}) was carried out for identical initial contamination levels of all soil samples with Paraoxon (630 mg kg^{-1}_{soil}) or Diazinon (850 mg kg^{-1}_{soil}) or Parathion (185 mg kg^{-1}_{soil}) (Fig. 2).

Table 1. Characteristics of soil samples used in the experiments

Soil type	pH	Humidity, %	Humus, %
Sand	7.4±0.2	90±5	0.0±0.2
Gray forest	6.5±0.3	71±4	5.1±0.3
Chestnut	8.0±0.2	82±6	2.1±0.1
Chernozem	7.0±0.2	75±5	6.7±0.4

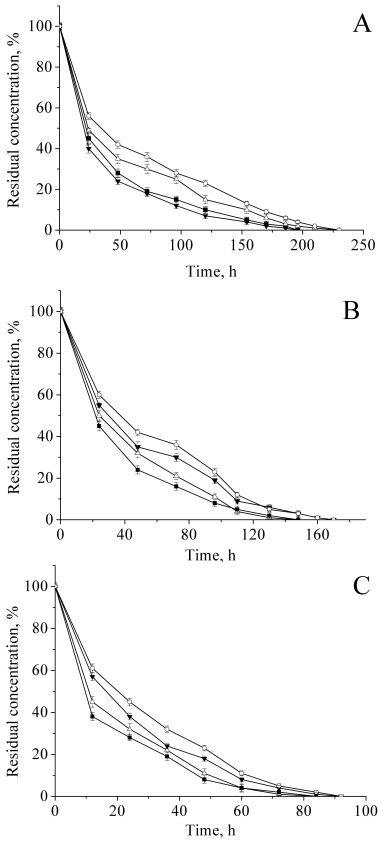

Figure 1. **Desrtuction of 630 mg kg^{-1}_{soil} of Paraoxon (A) 850 mg kg^{-1}_{soil} of Diazinon (B), 185 mg kg^{-1}_{soil} of Parathion (C) under the action of enzyme immobilized on wheat straw: ■ – sand, Δ – chestnut soil, ▼ – grey forest soil, ○ – chernozem.**

The introduction of the immobilized enzyme was demonstrated to ensure 100% decomposition at 20°C within a maximum of ≈10 days for the specified pesticide concentrations in all soil types examined. In sand, pesticide hydrolysis occurred at the maximum rate for all three pesticides investigated, compared to the other soil samples tested. This may well be connected with the fact that in sand, unlike other soil types, the organic component (humus) is absent; meanwhile, the pesticides can interact with its components, becoming adsorbed on them and, consequently, unavailable for enzymatic decomposition. In chestnut soil, pesticide hydrolysis occurred faster than in other humus-containing soil types — possibly due to the high pH value, which is closest to the optimum pH for His_6-OPH enzyme action. The half-life of the active immobilized enzyme in soils under the specified conditions of OPC hydrolysis was 130 days (Fig. 2).

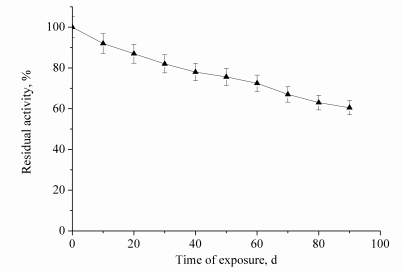

Figure 2. Residual activity of enzyme immobilized on wheat straw in sand.

CONCLUSIONS

The immobilization of His_6-OPH on a cellulose-containing carrier (straw) allowed to obtain a stable enzyme preparation that, introduced into soils contaminated with various OPC, ensured the decomposition of pesticides in high concentrations within a sufficiently short period of time. In addition, immobilization utilized a crude enzyme preparation. So this approach, undoubtedly, makes the use of a technically and technologically easy to produce and inexpensive immobilized preparation. Also the immobilized enzyme retains its catalytic activity in soil for a prolonged time.

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