

Impact of structural features of halloysite nanotubes on adsorption and catalytic properties of β -galactosidase

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Experimental

Samples: Three samples of halloysite nanotubes, further denoted as Hal-X, where X = 50, 30 and 12.5 – the average value of nanotube internal diameter in nm,^{S1} have been studied. The sample Hal-30 was purchased from Sigma-Aldrich Company, while Hal-50 and Hal-12.5 samples were provided by LLC «Molecular Technologies and New Materials».

Adsorbate: The adsorption of β -galactosidase from *Aspergillus oryzae* fungi (EC 3.2.1.23) was studied. This is a monomeric protein, the relative molecular weight (M_r) of which, according to various sources, ranges from 105^{S2} to 113 kDa.^{S3} The isoelectric point is 4.6.^{S4} The enzyme is promising for obtaining lactose-free milk formulas and oligosaccharides.^{S5,S6} A lyophilized preparation (Sigma-Aldrich, CAS 9031-11-2) was used in this study. The protein content in the preparation, determined by us using the Bradford assay,^{S7} was 8 %.

Methods: X-ray diffraction analysis (XRD) was performed at room temperature using a Tongda TD-3700 powder diffractometer (CuK α radiation; θ – θ geometry; 40 kV/15 mA generator; Mythen detector). The XRD patterns of halloysite samples were recorded in a 2θ Bragg angle range of 5–50° with a step of 0.05°. The ICDD-PDF2 powder pattern database was used for phase identification.

The surface morphology was examined by scanning electron microscopy (SEM) on a SEM-69-LV electron microscope (BiOptik, Russia) with a magnification of $\times 500$ to $\times 20\,000$ at an accelerating voltage of 30 kV. The particle size distribution for each sample was obtained based on manual measurements. The length of the edge at the square base was used as a parameter characterizing the size of cylindrical halloysite nanotubes.

Low-temperature nitrogen adsorption isotherms were recorded on an ASAP-2020 automatic sorbometer (Micromeritics, USA). Prior to measurement, the samples were degassed (350 °C, 4 h). Brunauer–Emmett–Teller (BET) method was applied for surface area analysis, while the micropore volume and corresponding surface area were calculated by the t-plot method. The mean internal pore diameter and total pore volume were determined by Barrett–Joyner–Halenda (BJH) method.

The amount of acid sites in the zeolite samples was identified by ammonia temperature-programmed desorption (NH₃-TPD) using a USGA-101 (UNISIT, Russia) universal sorption gas analyzer equipped with thermal conductivity detector. A weighed sample was placed in a quartz reactor, then heated in helium to 500 °C, annealed in helium at this temperature for 1 h, and cooled to 60 °C. Ammonia saturation was carried out in a dry NH₃/N₂ (1 : 10) flow at 60 °C for 15 min. Physically adsorbed ammonia was removed by holding at 100 °C in a dry helium flow for 1 h. The sample was then heated to 800 °C at a rate of 8 °/min.

Thermogravimetric and differential thermal analysis of the samples before and after enzyme adsorption were performed on SDT Q600 devices from TA Instruments. A sample weighing approximately 20 mg was calcined in a flow of air (100 ml/min) at a temperature rise rate of 10 °C/min.

Adsorption measurements: Enzyme adsorption was carried out from aqueous solutions at a temperature of 6 °C for 3-5 days. 5 ml of enzyme solution with concentrations from 20 to 1000 µg/ml (0.25 - 12.5 mg/ml for the preparation) were added to 100 mg of halloysite and left in the refrigerator. The adsorption value was determined by the difference in enzyme concentrations before and after the end of adsorption. After adsorption was complete, the samples were separated, washed several times with water, 5 ml of distilled water were added and left in the refrigerator for 5 days to check the reversibility of adsorption. The enzyme desorption value from the halloysite surface did not reach 2 %.

Determination of the β -galactosidase concentration: When determining the content of β -galactosidase in the solution, its catalytic activity in the hydrolysis reaction of 2-nitrophenyl- β -D-galactopyranoside (ONPG, synthetic enzyme substrate, Sigma-Aldrich, CAS 369-07-3) was studied. Initially, calibration curves were plotted taking into account that the initial rate of substrate hydrolysis is proportional to the content of active enzyme. The rate of the enzymatic reaction was determined by the rate of accumulation of 2-nitrophenol (colored reaction product) by absorption at $\lambda = 420$ nm using a JENWAY 6310 spectrophotometer. The reaction was carried out in plastic cuvettes (1 cm) in phosphate buffer (pH 7.5) at a saturating concentration of the substrate (3 mM). The sample volume of the enzyme solution during the analysis was 0.05 or 0.1 ml.

Determination of enzyme activity on the surface of halloysite nanotubes: Samples (10–20 mg) after enzyme adsorption were placed in plastic cuvettes ($l = 1$ cm), a substrate solution in a phosphate buffer pH 7.5 was added, and the rate of 2-nitrophenol accumulation was determined as described in the previous section. The catalytic activity of the enzyme was calculated in arbitrary units/s mg (protein).

Determination of the parameters of the Michaelis–Menten equation: The Michaelis–Menten equation describes the best-known kinetic model of an enzymatic reaction. The Michaelis constant (K_m) characterizes the affinity of the enzyme and substrate, and the maximum velocity (V_{max}) characterizes

the enzyme activity. To determine these parameters, the dependence of the reaction rate on the substrate concentration in the range of 0.2–3.3 mM was studied (Figure S1).

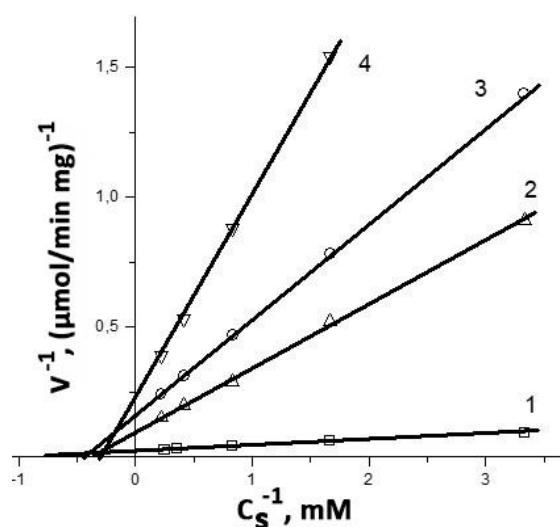


Figure S1 Determination of parameters in the Michaelis–Menten equation for β -galactosidase in solution (1) and on the surface of Hal-50 (2), Hal-30 (3) and Hal-12.5 (4) samples.

Study of the enzyme thermal stability: Thermal stability of β -galactosidase in solution and on the halloysite surface was studied at a temperature of 59 °C. Heterogeneous sample portions, as well as enzyme solutions in water or phosphate buffer (pH 7.5) were kept in a thermostat in the time interval of 10 – 60 min, after which the catalytic activity was determined using the standard method. Experimental results were processed in the coordinates of the first-order kinetic equation to find the effective inactivation (denaturation) rate constants.

Results and Discussion

XRD patterns of all samples (Figure S2) contain peaks characteristic of halloysite, however in samples Hal-50 and Hal-12.5 some impurities like gibbsite or alunite can be observed. Nevertheless, the amount of these phase impurities does not exceed 10 wt.%.

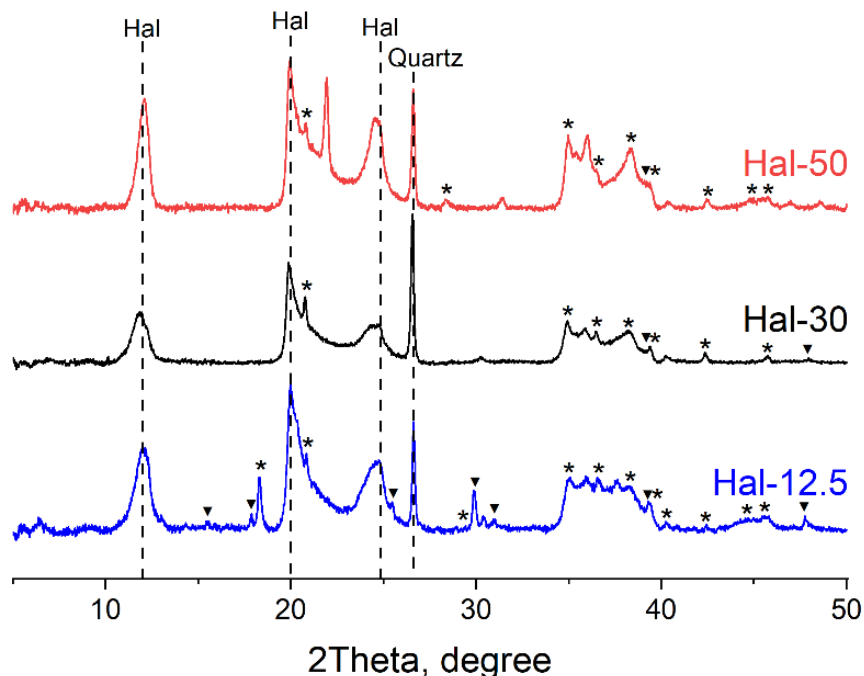


Figure S2 XRD patterns of halloysite nanotubes under study (Hal – PDF 29-1487, Quartz – PDF 05-0490, * - Alunite – PDF 72-1630, ▼ – Gibbsite – PDF 76-1782).

Figure S3 shows micrographs of halloysite samples obtained with a SEM-69-LV 2023 scanning electron microscope. These images show that in addition to tubular structures, the samples contain spherical and plate structures, as well as their agglomerates. This is especially characteristic of samples Hal-12.5 and Hal-50. The mean length of the tubes is 1.5 μm .

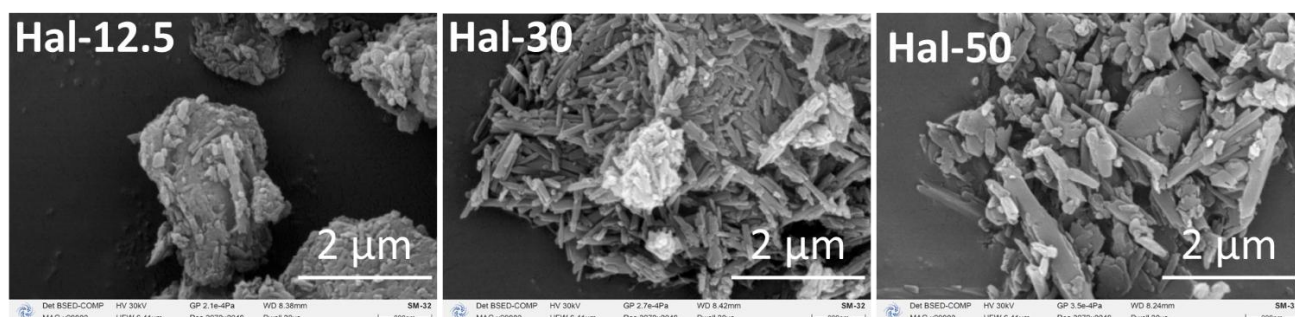


Figure S3 SEM images of samples under study.

Immobilized enzyme shows thermal stability under air up to 225 $^{\circ}\text{C}$ (Figure S4), which is similar to pristine β -galactosidase.

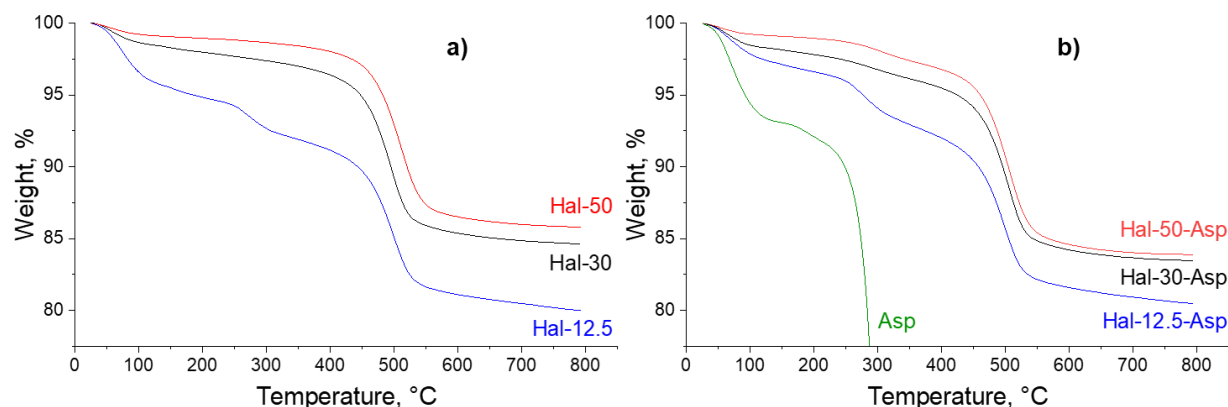


Figure S4 Thermogravimetric analysis of halloysite nanotubes before (a) and after (b) enzyme immobilization.

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