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Studying major kinetic relationships of xylan hydrolysis by a free and immobilized trichoderma viride enzyme complex

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Abstract

Today oligosaccharides are widely used in food, drug and cosmetics production. Enzymatic hydrolysis of polysaccharides is one of the prospective ways of oligosaccharide production. This work studies kinetic regularities of xylan hydrolysis using free and immobilized xylanase enzyme produced by *Trichoderma viride* (endo-1,4-*D*-xylanase, EC 3.2.1.8). The analysis of kinetic regularities and values of rate constants suggests a hypothesis about some peculiarities of the mechanism of xylan hydrolysis with a free and immobilized enzyme complex. The hypothesis on the insignificant effect of the immobilization on the enzyme complex activity was proposed. The synthesized catalytic system is reusable (at least 10 runs) and can be used for plant glycan hydrolysis to produce oligosaccharides of various polymerization degree.

Keywords: immobilization, enzyme complex, *Trichoderma viride*, kinetics.

Introduction

Presently oligosaccharides are widely used in food production. The main property of such carbohydrates is their positive effect on human organism through selective stimulation of useful intestine microflora growth. Indigestible oligosaccharides can lower a blood cholesterol level, improve the human immunity, promote fixation of mineral nutrients (iron, calcium, etc.), lower a cancer risk, improve a lipid metabolism. As for a non-food use of oligosaccharides, they are effectively used in the development of different bioimplants, medicine delivery systems, and in cosmetic industry.

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On an industrial scale, oligosaccharides are produced by four basic ways:

1) Plant polysaccharide hydrolysis.
2) Plant extraction (ineffective because of the great number of affecting factors).
3) Chemical synthesis.
4) Enzymatic synthesis from simple sugars by transglycosylation (Patel et al. 2011)

Special attention should be paid to biocatalytic degrading of heteropolysaccharide substrates with immobilized enzymes.

The kinetics of the enzymatic destruction of plant heteropolysaccharides is difficult to describe as polymer substrates provide enzymes with a wide range of attacking ways (in contrast to simple substrates having only one reaction center on a molecule). Catalytically active hydrolase groups are: serine –OH, cysteine –SH, aspartate or glutamate –COOH, as well as histidine imidasole. These groups function as nucleophilic catalysts to form intermediate compounds with the substrate (or as acid-base ones to promote splitting a proton from the water molecule attacking the bond to split, and linking that proton to the substrate group to leave). Metal-containing hydrolases metal atoms polarize the bond to split (by involving an H_2O molecule into its coordination sphere and promoting its ionizing).

Trichoderma fungi are one of the most actively studied microorganisms in the world as they can effectively produce many hydrolytic enzymes including xylanases. Its *Trichoderma viride* strain can produce several hydrolytic enzymes: β–glucosidase, pectin esterase and xylanase. Some researchers consider xylanase (endo-1,4-D–xylanase) the major enzyme in *Trichoderma viride*. It is a genuine xylan hydrolase, that is it has no cellulolytic activity and it is a low-molecular-weight peptide of molecular weight 18 kDa, pI 9.3. The main hydrolytic properties of xylanase from *Trichoderma viride* depend on the enzyme hydrolysis conditions, particularly the reaction time and substrate concentration. Xylanase from *Trichoderma viride* can hydrolyze xylans and their derivatives from different sources. Most often the hydrolysis products are oligoxylans, xylobioses and xylose,

where xylose is not the main product of xylanase, most often oligoxylans predominate in its hydrolizates.

The catalytic way of Trichoderma viride xylanase hydrolizing heteropolysaccharide substrates is not completely clear as this enzyme is very small and considerable hydrolytic effect can be achieved in a short space of time of incubation. The most popular hypothesis is that xylanase acts only on spatially accessible substrate sites to remove their oligoxylan and xylose fragments. The successive substrate transformation with Trichoderma viride xylanase can involve the following stages:

1) Splitting of some carbohydrate complexes and the formation of sugars small molecules which are easy to detach from the common chain.
2) Dissolving of oligomers – xylan derivatives formed.
3) Loosening of the xylan aggregates to form shorter oligoxylans, as well as di- and monosaccharides.

Today researchers have developed the method for xylanase immobilization on gluteraldehyde – activated aluminum oxide pellets (Sushi et al. 2012). Xylanase from *Bacillus pumilus* SV-85S was immobilized on polymethyl methacrylate nanofiber membrane. pH and temperature stability of xylanase was enhanced upon its covalent immobilization. The immobilized enzyme was active on repeated use and retained 80% of its initial activity after 11 reaction cycles (Pankaj et al. 2013). The method for *Armillaria gemina* xylanase immobilization onto SiO_2 nanoparticles was proposed. The immobilized enzyme provided 37.8 % higher production of xylooligosaccharides compared to a free enzyme (Saurabh et al. 2013). Xylanases from *Pholiota adipose* was covalently immobilized on functionalized silicon oxide nanoparticles. The immobilized enzyme provided 45 % higher concentrations of xylooligosaccharides compared to a free enzyme (Saurabh et al. 2012). *Aspergillus niger* xylanase was immobilized onto Fe_3O_4-coated chitosan magnetic nanoparticles prepared by the layer-by-layer self-assembly approach. The immobilized xylanase showed improved thermostability and storage stability compared with a free enzyme (Ming et al. 2014).

The detection and analysis of kinetic regularities of xylan hydrolysis would allow finding optimum reaction conditions to accumulate the maximum amount of physiologically active tri- or pentaoligosaccharides in the reaction environment. In the present paper for the enzymatic hydrolysis of xylan free and immobilized glycolytic enzyme complex from *Trichoderma viride* was used for the accumulation of physiologically active tri- and pentaoligosaccharides in the reaction medium.

Materials and Methods

Synthesis and analysis of catalysts

The heterogeneous catalytic system was synthesized in which its active component, a *Trichoderma viride* (Fluka) enzyme complex, was linked to the surface of a modified insoluble organic carrier - polymer SEPABEADS EC-HA 403 (Resindion SRL) – by molecular linking with multifunction reagents (phosphate buffers, glutaraldehyde). An infrared Fourier spectroscopy, low-temperature nitrogen adsorption and X-ray photoelectron spectroscopy were applied to study the physicochemical properties of the optimum catalytic system.

Kinetics

A reciprocating swinging reactor (to exclude external diffusion limitation) was used in the kinetic experiments. Sampling was seven hours long. The initial polysaccharide concentration varied from 0.07 to 4 g/l. The amount of the enzyme was 3560 e.u. To study the stability of the catalytic system obtained, successive heteropolysaccharide hydrolysis experiments were conducted. The synthesized biocatalytic system was used to hydrolyze plant heteropolysaccharides such as xylan and glycans of lint (Linum usitatissimum) and valerian (Valeriana officinalis). Xylane hydrolyzate was analyzed using liquid chromatography with refractive index (RI) and mass(MS) detection. Liquid phase chromatograph ULTIMATE 3000 equipped with RI detector, API-2000 mass spectrometer and 50 cm staleness steel column field with Reprogel-H -9μ sorbent was used for the analysis. The 10 mM solution of sulfuric acid in deionized water was used as eluent. The liquid phase flow rate was maintained 0.5 ml/min, the system pressure was 80 Bar, colomn temperature was maintained at 30 ^{0}C. The chromatograms were evaluated by area normalization method. All the chemicals were of analytical grade.

Results and Discussion

In Fig.1 typical chromatogram of hydrolyzed xylan solution is presented.

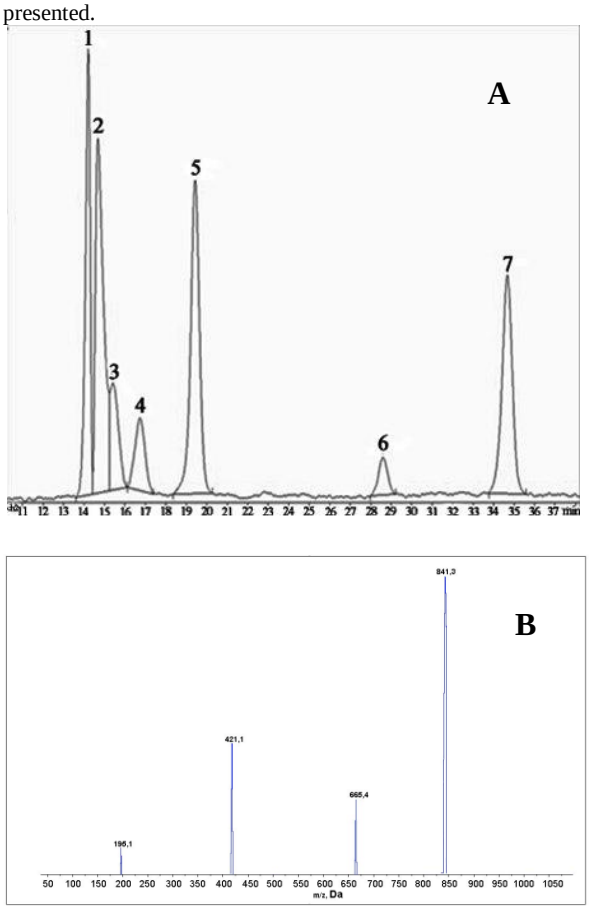


Figure 1: a) Refractive index chromatogram of hydrolyzed xylan (1-pentasaccharide, 2-tetrasaccharide, 3-trisaccharide, 4-disaccharide, 5 – galacturonic acid, 6 –xylose, 7 – glyceryne, b) Mass spectrogram of pentasaccharide

The solution contains penta-, tetra-, tri- and disaccharides, galacturonic acid, xylose and glyceryne. The typical MS spectrum of the obtained pentasacharide is presented in the figure, the ion lines can be easily attributed to pentaxylanes.

Native and immobilized enzyme complex activities were compared using xylan hydrolysis.

To choose the best hydrolysis temperature and pH some experiments were carried out in the temperature and pH ranges of 20ºC to 60ºC and 3 to 8 respectively.

As it is seen from the data above, the immobilization did not change the optimum enzyme activity conditions (t=40ºC and pH 4.8) but improved the temperature and pH stability of the catalyst (Fig.2).

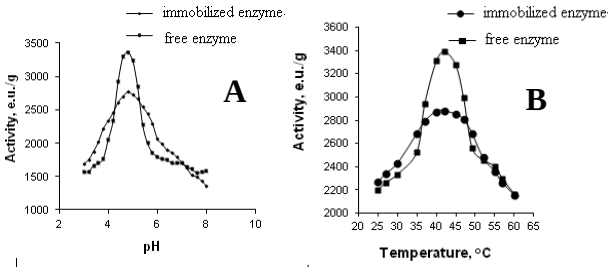


Figure 2: Free and immobilized *Trichoderma viride* enzyme complex activities as a function of: a) reaction medium temperature; b) reaction medium pH (kinetic method)

To evaluate the kinetic parameters of xylan hydrolysis we experimented on varying the initial substrate concentrations and amounts of free enzyme and synthesized catalyst at different temperatures and pH. The primary kinetic curves are shown in Figures 3 and 4.

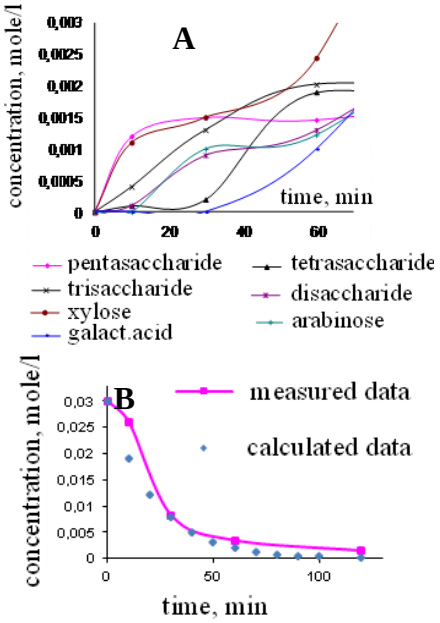


Figure 3: Catalysate composition after xylan hydrolysis with the enzyme complex (pH 4.8, t=40 ^{0}C, $m_{enzym\ prep}$= 0.42 g, $V_{react.mix}$= 15 ml): a) accumulation of hydrolysis products, b) initial polysaccharide substrate conversion.

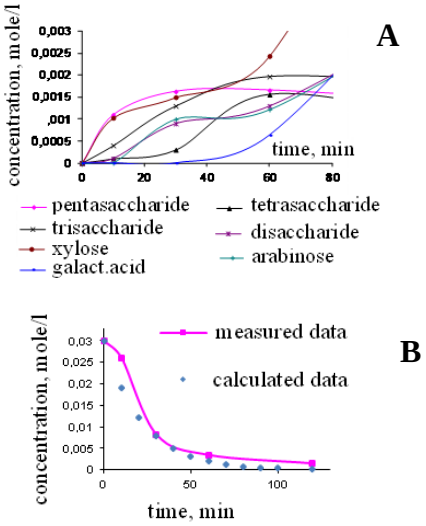


Figure 4: Catalysate composition after xylan hydrolysis with the catalyst (pH 4.8, t=40 ^{0}C, C_{cat} = 0.6 g, $V_{react.mix}$ = 15 ml): a) accumulation of hydrolysis products, b) initial polysaccharide conversion.

The proportion of individual fractions of oligosaccharides and monoses in the catalysates suggests that immobilization did not change the distribution of activities in the enzyme complex.

Since the sum of all the products and initial xylan was not constant in both experimental series, we took only reaction products sum as input data for calculations (see Fig. 5). The current concentration of the initial material ($[poly]$) was evaluated as

$$[poly] = [0.03 - \Sigma(\text{all products})]\ \text{mole/l} \qquad (1).$$

Fig. 5 shows a calculated curve (formation of all the reaction products in xylan hydrolysis) obtained by the integration of equation

$$-d[poly]/dt = +k_{\Sigma}[poly] = d[all\_products]/dt \qquad (2)$$

at $k_{\Sigma} = \Sigma[k_i]$ =0.008 min^{-1}

Where $\Sigma[k_i]$ is the sum of constants of parallel formation of all possible oligomers and monosaccharides from the initial polymer. The catalyst effectiveness is implied in the values of rate constants k_i.

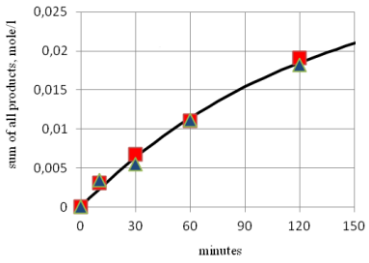


Figure 5: Sum of all the hydrolysis products in the systems of xylan/free enzyme (red squares), xylan/catalyst (blue triangles) and the calculated curve of their formation

Fig. 5 data show that

1) xylan hydrolysis on the catalysts selected can be described as a first-order reaction with respect to the initial xylan;
2) the consumption of the initial xylan on the catalysts selected (corrected for their concentrations) has the same rate.

The kinetic curves of the formation of all the products (see Fig. 3) suggest that the initial rates of the formation of practically all metabolites are zero – except pentooligomers and, unexpectedly, such monosaccharide as xylose. This fact allows supposing that separation of the above products from the major polymer mass is the primary stage of the xylan hydrolysis, that is two parallel reactions occur.

$$d[poly]/dt = -(k_1 + k_2)[poly] \quad (3)$$

$$d[penta + all\_its\_transformation\_products]/dt = k_1[poly] \quad (4)$$

$$d[xylose]/dt = k_2[poly] \quad (5)$$

In this case, all the other mono- and oligosaccharides formation is secondary reactions of the further transformation of pentasaccharide because their formation from xylose is hardly possible.

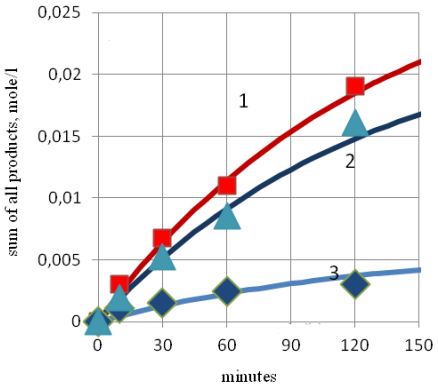


Figure 6: Calculated curves and experimental points for: (1) the sum of all hydrolysis products in the system of xylan/free enzyme; (2) the sum of all hydrolysis products – but xylose – in the system of xylan/free enzyme; (3) xylose concentration in the reaction medium during the reaction.

The calculated curves and experimental points (for the sum of all hydrolysis products in the system of xylan/free enzyme – curve 1, the same sum without xylose – curve 2, the kinetic curve of xylose formation – curve 3) shown in Fig. 6 agree well to each other. The reliability of approximation (R^2 evaluated by the standard Excel 93_2003 software) for the linear correlation between the measured and calculated values of the parameters under analysis was R^2 = 0.97 for the following rate constants: k_Σ=0,008 min^{-1}, k_1=0,0064 min^{-1} and k_2=0,0016 min^{-1}.

In the hydrolysis tetra- and trisaccharides are also formed and further spent, as their concentrations grow first, but lower later with time. Disaccharides (arabinose, xylose and galacturonic acid) appear to be the end-products of hydrolysis in the chosen reaction conditions. Their concentration profiles have no maximums. Xylan hydrolysis flowchart obtained for this stage can be shown as follows (Fig.7):

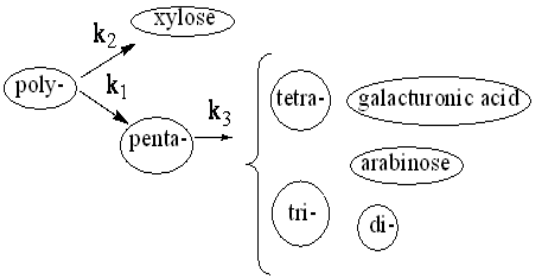


Figure 7: Flowchart of xylan hydrolysis

The further investigation requires the evaluation of constant k_3, that is pentaoligomer consumption constant. The ways of tetra- and trisaccharide formation can be determined from the values of the corresponding constants in modeling the flowchart (Fig.7).

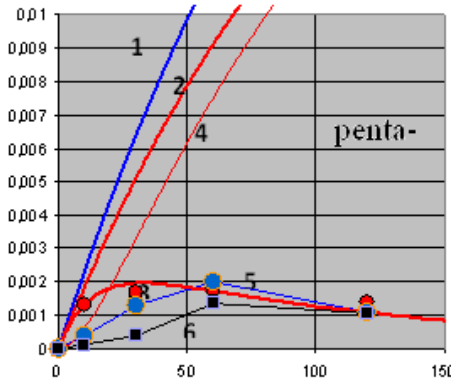


Figure 8: Calculated curves and experimental points: (1) the sum of all the hydrolysis products in the system of xylan/free enzyme; (2) the sum of all the hydrolysis products – but xylose – in the system of xylan/free enzyme; (3) pentasaccharide concentration in the reaction medium during the reaction; (4) the curve of the formation of all pentasaccharide transformation products; (5) experimental points of trisaccharide formation and depletion; (6) experimental points of tetrasaccharide formation and consumption.

Pentasaccharide concentrations profile is well described by the system of successive first-order reactions:

$$d[penta]/dt = k_1[poly] - k_3[penta] \quad (6)$$

at k_1=0,0064 min^{-1} , k_3 = 0,075 min^{-1}.

Fig. 8 shows experimental points and a calculated curve (3) of pentaoligomers formation and consumption. The same figure shows the calculated curve of the formation of the sum of all possible pentaoligomer transformation products (curve 4). Initially, the curve slope corresponds to pentaoligomer consumption rate. However, it can be seen that it is significantly higher than tetra- and trioligomers formation rate. Figure 8 shows experimental points of tri- and tetraoligomers formation and consumption – curves 5 and 6 respectively.

If we suppose that tri- and tetraoligomers production occurs in parallel in the hydrolysis of pentaoligomers, then these processes should be described by equations:

$$d[penta]/dt = k_1[poly] - k_3[penta] \quad (7)$$

$$d[tri]/dt = k_{31}[\ penta\ ] - k_4[tri] \quad (8)$$

$$d[tetra]/dt = k_{32}[\ penta\ ] - k_5[tetra] \quad (9)$$

where $k_3 = k_{31} + k_{32}$, whereas k_4 and k_5 are the further transformation constants of tri- and tetrasaccharides respectively. Indeed, values k_3 = 0.075 min^{-1}, k_{31} = 0.05 min^{-1} and k_{32} = 0.025 min^{-1}, k_4 =*0.045* min^{-1} and k_5 =*0.03* min^{-1} allowed describing well tri- and tetrasaccharides formation and consumption (Fig.9).

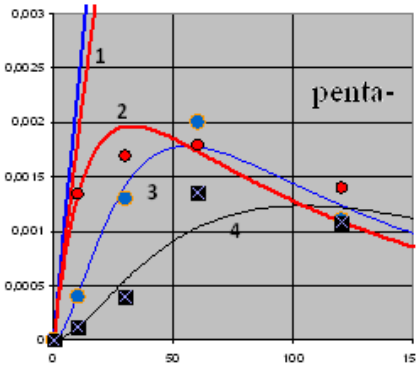


Figure 9: Calculated curves and experimental points points: (1) the sum of all the products of xylan pentaoligomer transformation; (2) pentasaccharide concentration profile in the reaction medium during the reaction; (3) trisaccharide concentration profile in the reaction medium during the reaction; (4) tetrasaccharide concentration profile in the reaction medium during the reaction.

It should be noted that the value of pentasaccharide consumption constant (k_{31}) equals the sum of tri- and tetrasaccharide semiproducts formation constants ($k_{31} = k_4 + k_5$). Consequently, it can be suggested that the above ways of pentasaccharide transformation are the only ones. Disaccharide can be formed only in one of further hydrolysis reactions. Some monosaccharides are formed both from pentasaccharide, simultaneously with tetra- and trisaccharides, and in the hydrolysis of the latter.

One should understand the sequence of oligosaccharide transformations into mono- and disugars. As seen from Equation 8, for example, tetrasaccharide is hydrolyzed further, and it is still unclear into which low-molecular-weight saccharides. Let us designate the latter as X. Their production is to follow Equation (10):

$$d[X]/dt = d[di]/dt = k_5[tetra] \quad (10)$$

It turned out that the calculated curve for the low-molecular-weight saccharide X production matches well disaccharides concentration profile (Fig. 8, curve 4), and the sum of tetra- and disaccharides is well described by equation (9a):

$$d[tetra - +disaccharides]/dt = k_{32}[penta] \quad (9a)$$

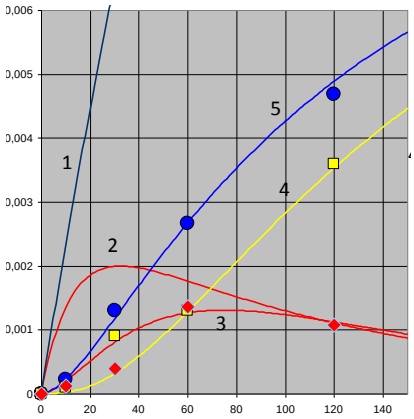
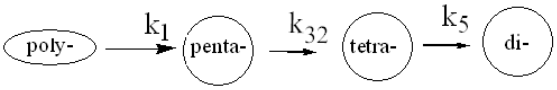


Figure 10: Calculated curves and experimental points: (1) the curve of the sum of all products of xylan transformation; (2) the calculated pentasaccharide concentration profile in the reaction medium during the reaction; (3) the calculated profile and experimental points of trisaccharide concentration in the reaction medium during the reaction; (4) the calculated profile of tetrasaccharide hydrolysis products and experimental points of disaccharide accumulation; (5) the calculated profile of hydrolysis products formation from pentasaccharide according to the reaction of its transformation into tetrasaccharide and experimental points of the sum of tetra- and disaccharides.

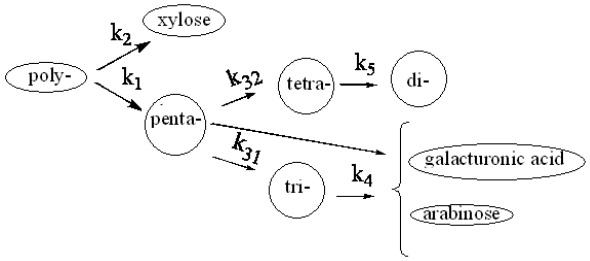
The agreement between the calculated and experimental values of its formation and transformation into disaccharide confirms the suggested way of transformation till the end product, disaccharide (Flowchart 1):



Flowchart 1

Thus, the flowchart of xylan hydrolysis with the enzyme complex (Flowchart 2) can be presented as follows:

Flowchart 2



However, it remains unclear which of the monomer sugars is produced at the stage of the further pentasaccharide hydrolysis to tri- and tetraoligomers as well as in trioligomer hydrolysis.

Figure 11 shows experimental points and calculated kinetic curves to illustrate the xylan hydrolysis flowchart suggested.

The suggested hydrolysis flowchart can be described by the system of differential equations:

$$d[poly]/dt = -k_\Sigma[poly] = -(k_1 + k_2)[poly] \quad (3)$$

$$d[xylose]/dt = k_2[poly] \quad (5)$$

$$d[penta]/dt = k_1[poly] - k_3[penta] \quad (6)$$

$$d[tri]/dt = k_{31}[penta] - k_4[tri] \quad (8)$$

$$d[tetra]/dt = k_{32}\| \ penta \ ] - k_5[\ tetra \ ] \quad (9)$$

$$d[di]/dt = k_5[tetra] \quad (10)$$

$$d([arabinose]+[galact.acid])/dt = \quad (11)$$

$$(1/5\times k_{32} + 2/5\times k_{31})[penta] + 3k_4[tri]$$

for $k_\Sigma = k_1 + k_2$, and $k_3 = k_{31} + k_{32}$,

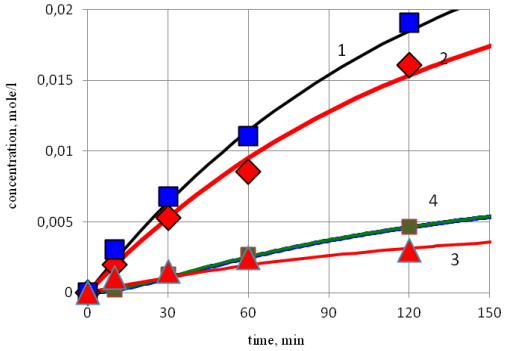


Figure 11: Calculated curves and experimental points: (1) the curve of the sum of all products of xylan transformation; (2) the concentration experimental points and calculated profile of all saccharides formed through pentasaccharide formation stage; (3) the calculated profile and experimental points of xylose concentration during the reaction; (4) the experimental points and calculated profile of the sum of tetra- and disaccharide concentrations.

The hydrolysis stage rate constants (min^{-1}) are given in the following table:

Constants	k_1	k_2	k_{31}	k_{32},	k_4	k_5
Free enzyme	**0.0064**	**0.0016**	**0.05**	**0.025**	***0.045***	***0.03***
Immobil.catalist	0.0064	0.0016	0.04	0.02	***0.04***	***0.03***

To describe xylan hydrolysis with the catalyst on the basis of immobilized enzyme complex, the sum of the reaction products only was taken as an input for the calculations, because the sum of all the products and the initial xylan was not constant for both experimental series at every time moment. The current concentration of the initial product was determined from equation 1.

As it has already been noted, the sum of all the hydrolysis products in the xylan/free enzyme and xylan/catalyst systems can be well described by a single kinetic equation and the same rate constant (equation 3). Moreover, the profiles of xylan hydrolysis products and its oligomers hydrolysis products are practically identical (Fig. 12 and 13).

The kinetic analysis confirmed the general flowchart of xylan hydrolysis on the both catalysts studied, with slight deviations in some reaction rate constants, which can be primarily observed in the secondary and tertiary hydrolyses.

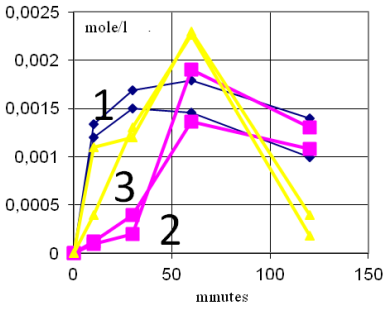


Figure 12: Concentration profiles of penta- (1), tetra- (2) and trioligomers (3) for xylan hydrolysis in xylan/free enzyme and xylan/catalyst systems.

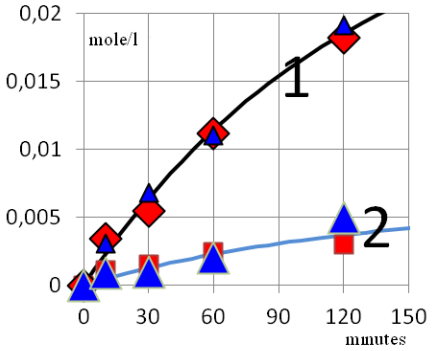


Figure 13: Calculated curves and concentration profiles of the sum of all xylan hydrolysis products (1) and xylose formation profile (2) in xylan/free enzyme and xylan/catalyst systems.

Conclusions

The analysis of kinetic regularities and values of rate constants suggests the hypothesis about some peculiarities of the mechanism of xylan hydrolysis with a free and immobilized enzyme complex: (1) immobilization has insignificantly affected the enzyme complex operation mechanism - which is confirmed by the same ratio of the rate constants before and after immobilization; (2) hydrolysis probably occurs both successively and parallel; (3) xylan hydrolysis has no inhibition by its products, which is probably due to the same monosaccharide nature of xylan and its hydrolysis products.
Basing on the analysis of kinetic regularities and values of velocity constants, the hypothesis on the insignificant effect of immobilization on the enzyme complex activity was proposed. The synthesized catalytic system is reusable (at least 10 runs) and can be used for plant glycan hydrolysis to produce oligosaccharides of various polymerization degree.

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