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# Biocatalytic process for synthesis of oxidized xylooligosacharides from xylan

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Pyrroloquinoline quinone dependent glucose dehydrogenases (PQQ-GDH) are attractive catalysts because of their wide substrate specificity and non-diffusible cofactor. PQQ-GDH from *Acinetobacter* sp. is capable to oxidize xylobiose or xylotetraose to the corresponding aldonic acid. The PQQ-GDH covalently immobilized on silica gel by cross-linking with glutaraldehyde exhibits higher thermostability compared with the free enzyme. The procedure of dichlorophenol indophenol mediated oxidation of xylobiose and xylotetraose in the presence of immobilized PQQ-GDH and laccase was developed.

**Key words:** xylooligosacharides, xylanase, pyrroloquinoline quinone dependent glucose dehydrogenase, aldonic acids

## INTRODUCTION

Xylooligosacharides (XOS) are sugar oligomers made up of xylose units, which are found in various plants [1]. The structures of XOS can vary in degree of polymerization, types of linkages (usually β-(1→4)-linkages) and modifications including other monomeric units. The number of xylose residues in XOS can vary from 2 to 10. XOS are produced from xylan containing lignocellulosic materials by chemical methods, direct enzymatic hydrolysis [2–4] or a combination of both treatments [5]. Production of XOS with chemical methods can be accomplished by steam, diluted solutions of mineral acids, or alkaline solutions. To produce XOS with chemical and enzymatic methods, xylan is generally extracted with an alkali from suitable lignocellulosic materials and extracted xylan is converted to XOS by xylanase. The enzymes having low exo-xylanase and / or β-xylosidase activity are usually applied [6]. Comparing to chemical treatment, enzymatic hydrolysis does not produce undesirable by-products or high amounts of monosaccharides and does not require special equipment.

Further modification of XOS is highly demanded by industry since the modifications can alter the rheology or biological properties of corresponding oligomers. Recently, it has been reported that acidic xylooligosaccharide in which glucuronic acid is linked to xylooligosaccharide by α-1, 2 bonds [7] promotes recovery from iron deficiency anemia by enhancing serum iron level [8]. Moreover, for example, oxidation of oligosaccharides can be an initial step to subsequent esterification or amination of hydroxyl groups. Various oxidizing reagents including 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), a common reagent for this purpose, are used to convert primary hydroxyl groups of oligo- and polysaccharides to carboxylic acids [9, 10]. However, an enzymatic oxidation opens ways for regioselective

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modification of highly functionalized carbohydrates without any protection / deprotection steps. Moreover, a formation of undesirable products is much lower compared to the chemical methods. Carbohydrate oxidases such as glucose oxidase and pyranose oxidase can catalyze the oxidation of the primary hydroxyl, secondary hydroxyls, or anomeric carbon hydroxyl of mono- and disaccharides to an aldehyde, ketone, or a lactone, respectively [11]. Galactose oxidase can attack oligosaccharides with terminal galactopyranosyl units [12]. Oligosaccharide oxidases that oxidize C1 hydroxyl groups of β-1,4-linked sugars can be used to derivatize xylan [13–17].

Pyrroloquinoline quinone (PQQ) dependent glucose dehydrogenases (PQQ-GDH) are promising biocatalysts that are able to oxidise a variety of mono- and disaccharides to the corresponding lactones [18–20]. Moreover, PQQ-GDHs can donate electrons to artificial electron acceptors including the direct electron transfer to the electrode surface [21–25] allowing a creation of bioelectrochemical conversion systems for carbohydrates. However, an activity of PQQ-GDH on XOS has not been analysed previously.

In this paper a procedure of enzymatic preparation and oxidation of xylooligosacharides by using xylanase, PQQ-GDH and laccase is presented.

## EXPERIMENTAL

### Materials

All chemicals used in the study were commercial products of guaranteed grade. Acetone, n-butanol, methanol, chloroform, acetic acid, 2,2'-azino-bis(3-ethylbenzthiazoline)-6-sulfonate (ABTS), dichlorophenolindophenol (DCPIP), and phenazine methosulphate (PMS) were from Sigma-Aldrich. Xylan from birch wood, spherical silicagel (pore diameter 260–340 Å), glutaraldehyde, and aminopropyltriethoxysilane (APTS) were from Fluka. Pre-coated aluminium sheets for TLC were from Macherey-Nagel.

### Enzymes

The xylanase (XylTSK) encoding gene was amplified by PCR from the chromosomal DNA of *Geobacillus* sp. TSK using the forward primer (ATGCGGAACGTCGTGCGTAAACC) and the reverse primer (TCACTTATGATCGATAATAGCCC). The purified PCR product 1.2-kb was cloned into the pTZ57R/T vector with an InsTAclone PCR Cloning Kit (ThermoFisher, Lithuania) for sequencing. 29 amino acids from N-terminus of xylTSK gene were identified as a signal peptide for protein secretion. A new pair of primers (GCACATATGGCGAAGAATGCAGATTCCTAT and CAGCTCGAGCAGTCACTTATGATCGATAATAGC) was used to obtain the xylTSK gene without signal peptide sequence. The amplified 1.1-kb DNA fragment was cloned into the pTZ57R/T vector to obtain pTSK13. The plasmid pTSK13 was then digested with *NdeI* and *XhoI*, and the gene encoding xylTSK was ligated into a pET-28b(+) vector (Novagen) to construct the expression plasmid pTSKBS12 for the production of the protein. *Escherichia coli* BL21(DE3) cells were transformed with pTSKBS12. The cells were grown in the Nutrient Broth medium (Oxoid, UK) containing kanamycin (50 μg $ml^{-1}$) at 30 °C until the absorbance at 600 nm reaches 1.0. Protein expression was induced with 1 mM isopropyl ß-D-1-thiogalactopyranoside, and the cells were allowed growing at 30 °C for additional 18 h. Cells were collected by centrifugation (30 min; 3,000 g), washed with 5 mM Tris/HCl buffer, pH 7.5, and disrupted by ultrasonic desintegrator. Cells debris was separated by centrifugation and a clear supernatant was applied on the carboxymethyl Sepharose (CM FF, GE Healthcare) column, preequilibrated with 5 mM Tris/HCl buffer, pH 7.5. Xylanase was eluted with a linear gradient of 0–1.0 M KCl in the same buffer. Fraction with xylanase activity was collected, concentrated by ultrafiltration (30 kDa cut-off) and stored at –20 °C. The specific activity of purified xylanase was 114 U/mg of protein.

The PQQ-dependent glucose dehydrogenase from *Acetobacter calcoaceticus* (GDH) was purified as described previously [26]. Laccase was purified from *Trichaptum abietinum* Lac2 according to the procedure described previously [25] and had the specific activity 750 U/mg of protein.

### Enzyme assay

GDH activity was determined at 30 °C by measuring the rate of discoloration of DCPIP at 600 nm in a 1 ml mixture containing 50 mM potassium phosphate buffer, pH 7.3, 0.03 mM DCPIP, 20 mM substrate and appropriate amount of GDH (0.02–0.1 U).

Xylanase activity was assayed in the reaction mixture containing 0.5% birchwood xylan, 50 mM Tris/HCl buffer, pH 7.5, and an appropriate amount of xylanase (0.02–0.1 U). Reaction was carried out for 10 min at 65 °C and reducing sugars were determined by the 3,5-dinitrosalicylic acid method using xylose as the standard [27].

Activity of laccase was determined by the oxidation of ABTS. The 1 ml of reaction mixture consisted of 50 mM sodium citrate buffer, pH 3.0, 0.25 mM ABTS and an appropriate amount of laccase (0.02–0.1 U). The ABTS oxidation was monitored by the increase of absorbance at 420 nm (ε = 36 000 $M^{-1}$ $cm^{-1}$) at 30 °C.

One unit of enzyme was defined as an amount of the enzyme that converts 1 μmol of substrate per min under the specified assay conditions.

### Immobilization of enzymes

Immobilization of enzymes was carried out on the spherical silicagel with a particle size 0.035–0.045 mm and pore diameter 260–340 Å. An amine group was introduced by boiling silicagel with 1% APTS for 1 hour. The probes of silicagel modified with APTS were activated with 1% glutaraldehyde at 30 °C for 30 min. Excess of glutaraldehyde was washed out with 50 mM Tris/HCl buffer, pH 7.5. 3–5 μg of laccase or 0.3 μg of PQQ-GDH were added per mg of the activated silicagel and immobilization was carried out by mixing at 30 °C for 30 min.

The unbound enzymes were desorbed with 50.0 mM Tris/HCl buffer, pH 7.5, containing 1.0 M NaCl and silicagel with the covalently immobilized enzymes was additionally washed with 50.0 mM Tris/HCl buffer, pH 7.5. The immobilized enzymes were stored in the same buffer at 4 °C for 1–2 months.

**Xylan hydrolysis and purification of xylooligosaccharides (XOS)**

For preparative hydrolysis, 25 ml of 2% birch wood xylan in 50 mM Tris/HCl buffer, pH 7.5, were treated with 2000 U of xylanase XylTSK at 65 °C for 4 hours. The reaction mixture was liophylised and residue was extracted with methanol at 65 °C overnight. Insoluble residue was removed, methanol was evaporated, and XOS were dissolved in water and separated on the Sephadex G10 column. Fractions were collected and XOS were analysed by TLC. The same compound containing fractions were combined, lyophilised and used for further experiments.

**XOS oxidation**

XOS oxidation was performed in 2 mM sodium acetate buffer, pH 5.5, at 30 and 50 °C for 1–6 hours in tightly closed tubes under mild agitation with 0.26–1.0 μmol of xylobiose 0.52 μmol xylotetraose, 2 U of PQQ-GDH with or without 2 U of laccase and 0.01–0.26 μmol of DCPIP. The probes were taken and products were analysed by TLC at appropriate time.

**Analytical methods**

XOS and oxidized XOS were analysed on pre-coated aluminium sheets. The running solvents consisted of *n*-butanol-acetone-water-acetic acid (4:5:1:1.5) for XOS mixture and xylotetraose / oxidized xylotetraose mixture; chloroform-ethanol-water (10:10:1.8) for xylobiose / oxidized xylobiose mixture. Liquid chromatography-mass spactrometry (LC-MS) analyses were performed using a high performance liquid chromatography system (CBM-20A controller, two LC-2020AD pumps, SIL-30AC auto sampler and CTO-20AC column oven; Shimadzu, Japan) equipped with a photodiode array (PDA) detector (SPD-M20A Prominence diode array detector; Shimadzu, Japan) and a mass spectrometer (LCMS-2020, Shimadzu, Japan) equipped with an ESI source. The chromatographic separation was conducted using a Hydrosphere C18 column, 4 × 150 mm (YMC, Japan), at 40 °C and a mobile phase that consisted of 5 mM ammonium acetate buffer, pH = 5.2 (solvent A), and acetonitrile (solvent B) delivered in the gradient elution mode at a flow rate of 0.6 mL $min^{-1}$. The elution programme used was as follows: isocratic 0% B for 0.5 min, from 0 to 60% B over 4.5 min, isocratic 60% B for 0.1 min, from 60 to 0% B over 0.1 min, isocratic 0% B for 5 min. Mass scans were measured from m/z 10 up to m/z 700, at 350 °C interface temperature, 250 °C DL temperature, ±4,500 V interface voltage, neutral DL/Qarray, using $N_2$ as nebulizing and drying gas. Mass spectrometry data was acquired in both the positive and negative ionization mode. The data was analyzed using the LabSolutions LCMS software.

## RESULTS AND DISCUSSION

**Xylanase from *Geobacillus* sp. TSK**

Searches in the databases revealed that the deduced amino acid sequence of xylanase XylTSK from *Geobacillus* sp. TSK showed extensive homology to xylanases belonging to family 10 glycosyl hydrolases as classified by Gilkes et al. [28]. The recombinant xylanase XylTSK was purified as described in Materials and Methods with the yield of 98.8% of total enzyme activity which reached 84800–94400 U/L of culture medium. The purified xylanase had specific activity of 540 U/mg and exhibited a band with a molecular weight of 45 kDa when SDS-PAGE was performed (data not shown). Based on the effect of temperature on the xylanase activity, the XylTSK exhibited optimal activity at 80 °C. Thermostability assays indicated that the XylTSK maintained full activity at 65 °C for 24 hours and retained 45% activity at 70°C for 1 hour. The optimum pH of the XylTSK was found to be 7.0 ± 0.5. The enzyme showed similar thermostability and temperature optimum as the xylanase from *Geobacillus stearothermophilus* [29] and *Geobacillus thermodenitrificans* JK1 [30].

**Analysis of hydrolysis products from birch wood xylan**

The xylanase XylTSK was applied for xylan hydrolysis. XOS were produced by degrading birch wood xylan in the presence of the XylTSK at 65 °C during 1–24 hours. The hydrolysis products were analysed by TLC. Prolonged hydrolysis of substrate with xylanase resulted in complete degradation of xylan and formation of a mixture of saccharides (Fig. 1). The XOS were extracted from the mixture and individual compounds were purified as described in Methods (Fig. 1). Molecular ion mass of each purified saccharide determined by mass spectrometry after HPLC separation corresponded to xylose (X1, $M_r$ calculated: 150, detected: $[M+Na]^+ = 173$), xylobiose (X2, $M_r$ calculated: 282, detected: $[M+NH_4]^+ = 300$,

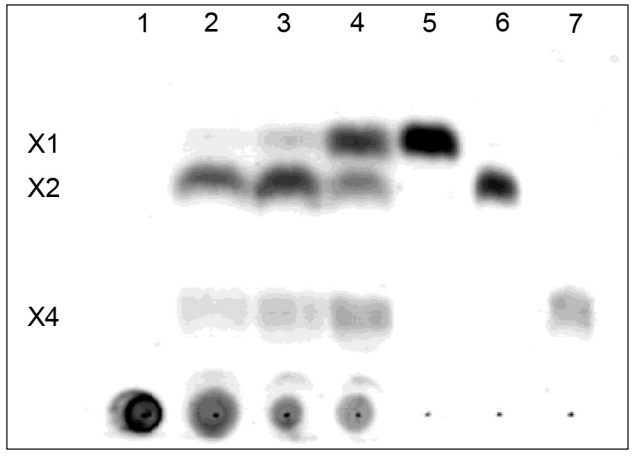


**Fig. 1.** TLC analysis of hydrolysis products of birchwood xylane by the recombinant xylanase from *Geobacillus* sp. TSK (1–4) and the purified xylooligosaccharides (5–7). Lane 1 – untreated xylan, lanes 2–4 – the products of xylan hydrolysis in the presence of xylanase after incubation at 65 °C for 1, 6 and 24 h, respectively. Lane 5 – xylose, lane 6 – xylobiose, lane 7 – xylotetraose

$[M+K]^+$ = 321, $[M–H]^-$ = 281, $[M+CH_3COO]^-$ = 341 and $[2M–H]^-$ = 563) and xylotetraose (X4, $M_r$ calculated: 546, detected: $[M+NaCl+NH_4]^+$ = 622, $[M+NaCl+K]^+$ = 643 and $[M+NaCl–H]^-$ = 603) as published previously [31]. The major end product of enzymatic conversion using XylTSK was xylobiose, however, neither xylotriose nor xylopentose was observed in contrast to xylanases from related microorganisms [32–34]. Moreover, xylose was formed after xylan conversion by XylTSK contrary to the xylanase from *Geobacillus* sp. 71 where xylobiose and xylotetrose were the major end products of the hydrolysis [35].

**Oxidation of XOS**

Since there were no data about PQQ-GDH ability to oxidize XOS, individual XOS were tested as a substrate for PQQ-GDH and its activity was determined as described in Methods. Both xylobiose and xylotetraose as well as xylose were substrates for PQQ-GDH. However, the activity of PQQ-GDH decreased when the length of XOS increased. Hence, the relative activity in the presence of 2 mM substrate decreased from 100% for xylose to 52 ± 9 % for xylobiose and further to 23 ± 3 % in the case of xylotetraose. The immobilized enzymes were removed from the reaction mixture by sedimentation and molecular ion mass of each oxidized saccharide was determined by mass spectrometry after HPLC separation. The bioconversion products corresponded to xylobionic (XB, $M_r$ calculated: 298, detected: $[M+NH_4]^+$ = 316, $[M+K]^+$ =337, $[M–H]^-$ = 297 and $[2M–H]^-$ = 595) and xylotetronic acid (XT, $M_r$ calculated: 562, detected: $[M+NaCl+NH_4]^+$ = 638, $[M+NaCl+K]^+$ = 659 and $[M+NaCl–H]^-$ = 619). It should be noted that under conditions used for HPLC, all oligosaccharides as well as the oxidized products appeared as ions of different adducts, a formation of which was discussed previously [36]. A tendency for the higher stability of ammonium adducts with increasing degrees of polymerization of oligosaccharides was also noticed [37].

The effectiveness of oxidation might be increased at higher reaction temperature. The native PQQ-GDH retained 95% of the initial activity after incubation at 50 °C for 1 hour and approximately 65% of the activity was observed after 24 hours, however, the enzyme was fully inactivated at 60 °C in 30 min. The immobilization of PQQ-GDH on silicagel increased its thermostability dramatically. The enzyme retained 100% and 73% of the initial activity after 1 hour incubation at 55 and 60 °C, respectively. The immobilized form of PQQ-GDH enabled the separation of the enzyme from the reaction mixture by centrifugation or simple sedimentation. This property was applied for the further bioconversions of XOS.

Oxidation of XOS by PQQ-GDH has resulted in reduction of the prosthetic group PQQ and subsequent reduction of the electron acceptor dichlorophenolindophenol (DCPIP). The full oxidation of XOS could be only achieved when an equimolar to substrate amount of DCPIP was used. Analysis of xylobiose bioconversion by PQQ-GDH in the presence of different amount of DCPIP showed that the oxidation of xylobiose with an equimolar amount of DCPIP finished rapidly resulting in the full oxidation of the substrate (Fig. 2). The reaction with ten and hundred fold lesser amount of DCPIP compared to the added xylobiose resulted in partial oxidation of xylobiose only (Fig. 2). That is non-applicable from a practical point of view. Laccases, enzymes with broad substrate specificity, capable to oxidize various phenolic compounds, might be used for re-oxidation of DCPIP [38]. A principal scheme of the DCPIP-mediated oxidation of XOS is shown in Fig. 3. The main problem to be solved was different

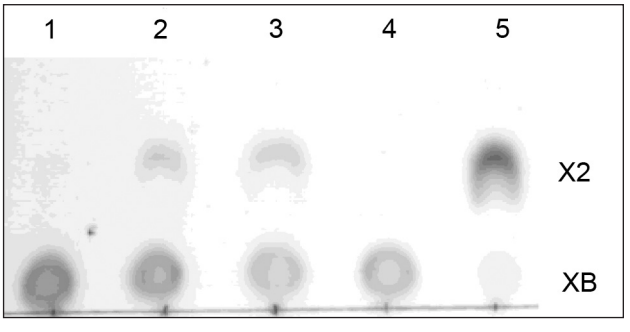


**Fig. 2.** TLC analysis of xylobiose (X2) oxidation using PQQ-GDH and varying amount of DCPIP and laccase. Reaction mixture consisted of 0.26 µmol xylobiose, 2 U of immobilized GDH and varying amount of DCPIP in 2 mM Na acetate buffer, pH 5.5. Additionally 2 U of immobilized laccase were added (lane 4 and 5). Lane 1 – 0.26 µmol DCPIP, lane 2 – 0.026 µmol DCPIP, lanes 3 and 4 – 0.0026 µmol DCPIP, lane 5 – without DCPIP. Reactions were performed at 50 °C for 1 hour. XB – xylobionic acid

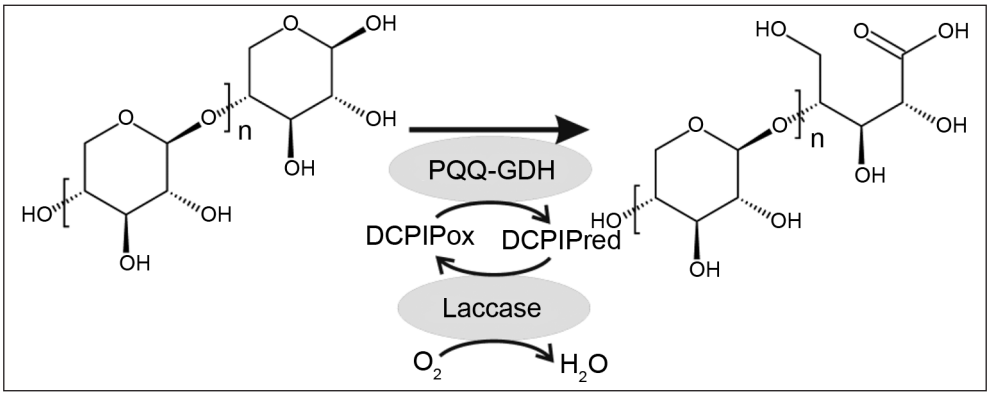


**Fig. 3.** A principal scheme of xyloologosaccharide oxidation. n – 0, 1, 3

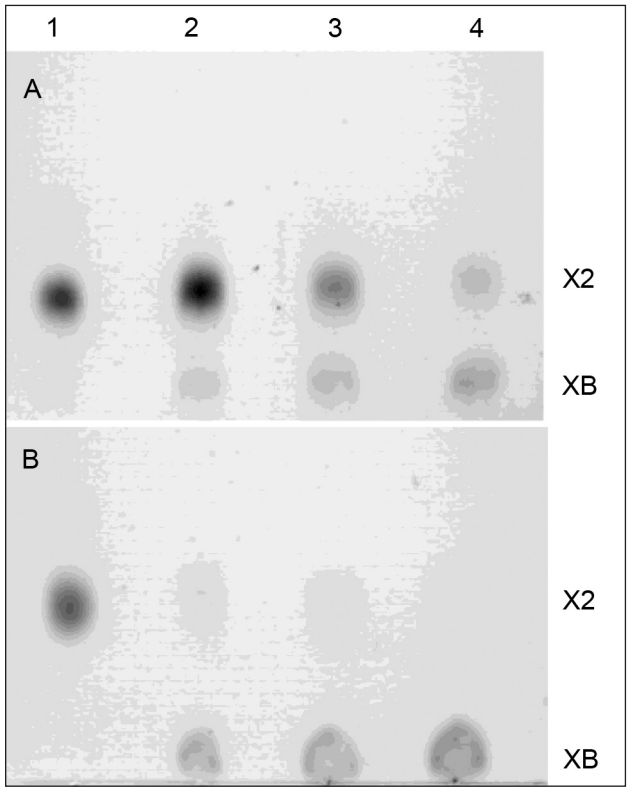


**Fig. 4.** TLC analysis of xylobiose (X2) oxidation by PQQ-GDH in the presence of laccase at 30 °C (A) and 50 °C (B). Reaction mixture consisted of 1 µmol of xylobiose, 2 units of PQQ-GDH, 2 units of laccase, 0.01 µmol DCPIP in 2 mM Na acetate buffer, pH 5.5. Reaction was performed for 1–4 hours. Lane 1 – xylobiose, lanes 2–4 – products of the reaction after 1, 2 and 4 hours of incubation, respectively. XB – xylobionic acid

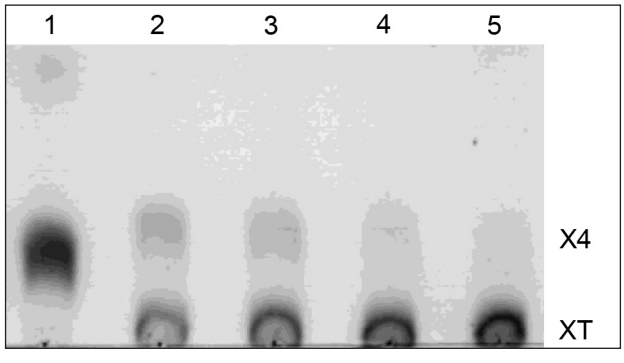


**Fig. 5.** TLC analysis of xylotetraose (X4) oxidation. Reaction mixture consisted of 0.52 µmol of xylotetraose, 2 units of each PQQ-GDH and laccase, 0.01 µmol DCPIP in 2 mM Na acetate buffer, pH 5.5. Lane 1 – xylotetraose; lanes 2 and 3 – reaction products after 2 and 4 hour incubation at 30 °C; lanes 4 and 5 – reaction products after 2 and 4 hour incubation at 50 °C. XT – xylotetronic acid

pH-optimum of laccase and PQQ-GDH. Most laccases usually oxidise substrates at acidic pH (2.0–4.0) while the activity dramatically decreases at more alkaline pH [39, 40]. PQQ-GDH oxidises substrates most effectively in the pH range from 5.5 to 9.0 [18]. Keeping in mind pH preferences of both enzymes oxidation of XOS and regeneration of DCPIP were carried out concurrently at pH 5.5, when both PQQ-GDH and laccase were still active. The addition of the immobilized laccase to the reaction mixture enabled a full oxidation of xylobiose in the presence of hundred fold lesser amount of the electron acceptor (Fig. 2). Similar results were obtained when xylotetraose was used as a substrate.

Analysis of dependence of xylobiose and xylotetraose oxidation on reaction temperature showed, as expected, that oxidation was more rapid at higher temperature and full oxidation of xylobiose and xylotetraose finished in 4 hours at 50 °C (Figs. 4, 5).

The developed simple procedure allows production of xyloaldonic acids from the corresponding XOS at gram-scale, however, pH control of the reaction mixture has to be carried out due to the formation of high concentrations of acidic product (data not shown). Keeping in mind that sugar acids are currently generating considerable interest because of their potential as probiotics or as platform chemicals and particularly their use as precursors in the manufacture of biomass derived plastics [41], the presented biocatalytic process opens new ways for the development of industrial processes including bioelectrocatalytic ones for production of xyloaldonic acids.

## CONCLUSIONS

PQQ-GDH from *Acinetobacter* sp. was capable to oxidise xylobiose and xylotetraose by forming the corresponding acids. The DCPIP-mediated oxidation of xylooligosacharides by PQQ-GDH and laccase was developed. By employing the immobilized enzymes and mediated oxidation the acidic XOS could be synthesized in good conversion yields.

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## BIOKATALIZINIS PROCESAS OKSIDUOTŲ KSILOOLIGOSACHARIDŲ SINTEZEI IŠ KSILANO

*S a n t r a u k a*

Nuo pirolochinolino chinono priklausomos gliukozės dehidrogenazės (PQQ-GDH) yra patrauklūs biokatalizatoriai, nes pasižymi plačiu substratiniu atrankumu bei savo sudėtyje turi stipriai surištą kofaktorių. Šiame darbe pirmą kartą parodyta, kad PQQ-GDH iš *Acinetobacter* sp. bakterijų oksiduoja ksilobiozę ir ksilotetraozę iki atitinkamų aldoninių rūgščių. PQQ-GDH, kovalentiškai imobilizuota ant silikagelio per glutaro aldehido jungtukus, buvo termostabilesnė už natyvų laisvą fermentą. Panaudojant imobilizuotas PQQ-GDH ir lakazę buvo sukurta dichlorofenolindofenolio medijuojama ksilobiozės ir ksilotetraozės oksidacijos procedūra.