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EVALUATION OF THE POTENTIAL OF FREE AND IMMOBILIZED THERMOPHILIC BACTERIAL ENZYMES IN THE DEGRADATION OF AGRO-INDUSTRIAL WASTES

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Abstract - Agro-industrial wastes are potential starting materials for the production of useful value-added compounds, including prebiotic oligosaccharides. In this paper, we evaluated the potential of thermophilic bacterial pectin- and xylan-degrading recombinant enzymes for the degradation of the agro-industrial wastes: apple pomace, wheat straw, wheat bran and distillers grains. For the immobilization of pectate lyase and xylanase, three different supports were used. The effect of enzyme immobilization was analyzed in terms of enhanced thermostability and activity against these wastes. For xylanase, the highest thermostability was achieved by immobilization on Sepabeads EC-EP/M. The best activity against bran and grains was obtained by immobilization on Sepabeads EC-HA/M. For pectate lyase, the highest thermostability was achieved by immobilization on Sepabeads EC-EP/M, however, activity against apple pomace pectin was slightly reduced by this immobilization. The length of oligosaccharides produced by both free and immobilized enzymes was also determined.

Key words: Thermophilic; agro-industrial wastes; pectate lyase; xylanase; immobilization

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INTRODUCTION

Prebiotics are generally defined as non-digestible polysaccharides and oligosaccharides, which promote the growth of beneficial lactic acid bacteria in the colon and exert antagonism to *Salmonella* sp. or *Escherichia coli*, limiting their proliferation (Patel and Goyal, 2012). An array of prebiotics with various origin and chemical properties exists. Some of them (inulin, fructooligosaccharides, galactooligosaccharides, lactulose) are recognized as established prebiotics, whereas other oligosaccharides (OS), including xylooligosaccharides (XOS), can be categorized as emerging prebiotics (Barreteau et al., 2006). XOS are characterized by various physiologically important actions such as reducing cholesterol, maintaining gastrointestinal health, and improving the biological availability of calcium. Besides, because they are moderately sweet, stable over a wide range of pH and temperatures, and can inhibit starch retrogradation, they improve the nutritional and sensory properties of food (Akpinar et al., 2007). Pectic oligosaccharides (POS) are also promising candidate prebiotics. Recent studies reported health benefits of pectins and POS, including regulation of lipid and glucose metabolism with decreased glycemic response and blood cholesterol levels, anticancer and immunological properties, anti-obesity effects, antibacterial and antioxidant properties (Ganan et al., 2010; Manderson et al., 2005; Rhoades et al., 2008).

OS can be obtained from the agricultural and industrial wastes that are abundant throughout the world; i.e., cheap wastes can be converted into useful value-added products. POS can be obtained from pectin-rich by-products such as sugar beet pulp, apple pomace or citrus peel (Concha Olmos et al., 2012; Ganan et al., 2010; Manderson et al., 2005; Martínez Sabajanes et al., 2012; Rhoades et al., 2008). XOS can be produced from corn cob, cotton and tobacco stalks, rice and wheat straw, hardwood or oat spelt (Akpinar et al., 2007, 2009; Falck et al., 2013; Wang et al., 2011). Microbial enzymes are the preferred choice for conversion of these wastes into OS. They provide high specificity, low energy or chemical consumption as well as low environment pollution (Sweeney et al., 2012). Thermostable enzymes have several generic advantages, allowing a reduced amount of enzyme needed because of higher specific activity and elongated hydrolysis time due to higher stability. In addition, thermostable enzymes are generally more tolerant and allow more flexibility in process configurations (Zhang et al., 2011). To the best of our knowledge, the potential of mesophilic but not thermophilic enzymes to be used in the production of OS from the wastes was mainly evaluated (Akpinar et al., 2007, 2009; Martínez Sabajanes et al., 2012; Wang et al., 2011).

In this paper, we report an evaluation of the potential of thermophilic microbial pectin- and xylan-degrading recombinant enzymes for the degradation of the agro-industrial wastes: apple pomace, wheat straw, wheat bran as well as distillers grains. The effect of enzyme immobilization on three different supporting materials was analyzed in terms of enhanced thermostability and activity against these wastes. The length of OS produced by both free and immobilized enzymes was determined.

MATERIALS AND METHODS

Identification of thermophilic enzymes associated with the degradation of pectin

Thermophilic polygalacturonic acid (PGA) enrichment culture was grown in the medium described by Takao et al. (2000). 1 g of soil was suspended in 100 ml of growth medium. The enrichment culture was cultivated for 24 h at 60°C at 180 rpm. 1 ml of the enrichment culture was transferred into fresh medium with the apple pomace instead of PGA and cultivation was repeated for 24 h. Cells were removed by centrifugation (7000×g, 20 min, 4°C), and solid ammonium sulphate was slowly added to the supernatant with constant stirring to achieve 80% saturation. The precipitate was recovered by centrifugation (12000×g, 20 min, 4°C), then dissolved in 50 mM Tris-HCl buffer (pH 7/60ºC). Extracellular protein samples were subjected to SDS-PAGE and zymographic analysis.

PGA zymography was performed in SDS/polyacrylamide gel containing 0.05% of PGA. After electrophoresis, the gel was washed for 20 min in 1% Triton X-100 and incubated in 50 mM Tris-HCl buffer (pH 7/60ºC) overnight at 60°C. After incubation, PGA zymogram was stained with 0.025% ruthenium red. SDS-PAGE was performed by the method of Laemmli (1970) on a 12% running gel, and protein bands were visualized by staining with the PageBlue™ Protein Staining Solution (Thermo Fisher Scientific). The zone of enzymatic activity in the zymogram was aligned with the corresponding band on the equivalent gel without substrate. The band was excised from the SDS/polyacrylamide gel and subjected to liquid chromatography-mass spectrometry (LC/MS) analysis. LC/MS was performed in the Mass Spectrometry Laboratory of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences. Acquired spectra of peptides were searched against bacterial protein database using the MASCOT (Matrix Science Ltd.) search algorithm.

Cloning and sequence analysis of thermophilic pectate lyase

In order to amplify the thermophilic pectate lyase gene, the primers P-1/2/3-PL47-F (5'- GAG ACG ATG GTA TAC GAA TAT ATT CTA GG -3') and P-1/2/3-PL47-R (5'- CTA ATG AAG ATT TCC CGC ACC CG -3') were designed. The *Bacillus* sp. TS-47 pectate lyase gene sequence AB045986 was used for

the construction of these primers. The primers were designed using the PRIMERSELECT component of LASERGENE 6 (DNASTAR). The pectate lyase gene was amplified in 50 µl of reaction mixture containing DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific), 0.25 µM each primer, and 10 ng of genomic DNA. Genomic DNA was extracted from the enrichment culture using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific). PCR was conducted under the following conditions: initial denaturation at 95°C for 2 min followed by 29 cycles each consisting of 95°C for 1 min, 60°C for 2 min, and 72°C for 3 min with a final extension step at 72°C for 7 min in an Eppendorf thermal cycler. Products of amplification were analyzed by electrophoresis through 1% agarose gel. PCR amplicons for the cloning of pectate lyase gene were prepared using the primers PL47-s-F2 (5'- CAT ATG GCT AGC ATG AAG GAA TTA GGG C -3') and PL47-R (5'- CGC AAG CTT CTA ATG AAG ATT TCC CGC -3'). A *Nhe*I site (underlined) was incorporated into the forward primer and a *Hind*III site (underlined) was incorporated into the reverse primer for cloning into pET-28c(+). PCR conditions were the same as stated above. PCR products were digested with *Nhe*I and *Hind*III (Thermo Fisher Scientific) according to the manufacturer's instructions. The pectate lyase gene was ligated into the pET-28c(+) vector, and the products were transformed into *E. coli* BL21(DE3). The sequences were aligned and analyzed using the MEGA 5.05 program (Tamura et al., 2011). Signal sequence prediction was performed using the PrediSi server.

Expression and purification of the recombinant pectate lyase

Transformants were grown in CASO broth (Merck KgaA) containing 30 µg kanamycin/ml. Protein expression was induced with 0.1 mM of isopropyl-β-D-thiogalactopyranoside when OD600 reached 0.5, and the incubation was continued for another 2 h at 37°C. Cultures were harvested by centrifugation (7000×g, 20 min, 4°C) and resuspended in AU6 buffer (20 mM Tris-HCl, 250 mM NaCl, 10 mM imidazole, 6 M urea, pH 7/20°C). Cells were disrupted by sonication, and the cell debris was removed by centrifugation at 12000×g for 20 min at 4°C. The resulting supernatant was dialyzed for 12 h at 4°C against AU2 buffer (20 mM Tris-HCl, 250 mM NaCl, 10 mM imidazole, 2 M urea, pH 7/20°C) and then subjected to affinity chromatography. The supernatant was loaded onto Bio-Scale™ Mini Profinity™ IMAC column (40 mm x 5.6 mm; Bio-Rad, USA) equilibrated in the buffer AU2. The column was washed with 25 mM imidazole, proteins were eluted with a linear 25 to 250 mM imidazole gradient in the same buffer at a flow rate of 1.0 ml/min. Active fractions were pooled and dialyzed for 12 h at 4°C against buffer BP (50 mM Tris-HCl, 10 mM imidazole, pH 9/20°C). The resulting protein solution was subjected to ion exchange chromatography. The solution was loaded onto a UNO Q6 column (12 x 53 mm; Bio-Rad, USA) equilibrated in the buffer BP. Recombinant pectate lyase was eluted with a linear 0 to 1 M NaCl gradient in the same buffer at a flow rate of 3.0 ml/min. Active fractions were pooled and dialyzed for 12 h at 4°C against buffer C (50 mM Tris-HCl, 10 mM imidazole, pH 7/60°C).

The molecular weight and purity of the recombinant pectate lyase were estimated by gel electrophoresis on a 12% running gel by the method of Laemmli (1970) under non-reducing conditions. Protein bands were visualized by staining with the PageBlue™ Protein Staining Solution (Thermo Fisher Scientific). Protein concentration was determined by Bradford's assay using bovine serum albumin as a standard (Bradford, 1976).

Removal of N-terminal His-tag from recombinant pectate lyase

N-terminal His-tag was cleaved by thrombin using the Thrombin Cleavage Capture Kit (Novagen) according to the manufacturer's recommendations. Affinity chromatography was used to eliminate uncleaved His-tagged pectate lyase. After chromatography, pectate lyase was dialyzed for 12 h at 4°C against 50 mM Tris-HCl buffer (pH 7/60°C). Both His-tagged and tag-free recombinant pectate lyase were used for activity analysis.

Purification of the recombinant thermophilic xylanase

We have previously reported identification, cloning, expression and characterization of the thermophilic endo-1,4-β-xylanase JK1 (Gerasimova et al., 2012). The purification protocol of JK1 was improved, scaled up and used in the current study. Induced culture was harvested by centrifugation (7000×g, 20 min, 4°C), washed twice and resuspended in ice-cold buffer A (20 mM Tris-HCl, 250 mM NaCl, 10 mM imidazole, pH 7/20°C). Cells were disrupted by sonication, and the cell debris was removed by centrifugation at 12000×g for 20 min at 4°C. The resulting supernatant was subjected to affinity chromatography. The supernatant was loaded onto a Bio-Scale™ Mini Profinity™ IMAC column (40 mm x 5.6 mm; Bio-Rad, USA) equilibrated in the buffer A. The column was washed with 20 mM imidazole, proteins were eluted with a linear 25 to 250 mM imidazole gradient in the same buffer at a flow rate of 1.0 ml/min. Active fractions were pooled and dialyzed for 12 h at 4°C against buffer BX (20 mM Tris-HCl, pH 8.4/20°C). The resulting protein solution was subjected to ion exchange chromatography. The solution was loaded onto a UNO Q6 column (12 x 53 mm; Bio-Rad, USA) equilibrated in the buffer BX. Xylanase JK1 was eluted with a linear 0 to 1 M NaCl gradient in the same buffer at a flow rate of 2.0 ml/min. Active fractions were pooled and dialyzed for 12 h at 4°C against 100 mM Tris-HCl buffer (pH 7/60°C).

The molecular weight and purity of the recombinant xylanase JK1 were estimated by gel electrophoresis on a 12% running gel by the method of Laemmli (1970) under non-reducing conditions. Protein bands were visualized by staining with the PageBlue™ Protein Staining Solution (Thermo Fisher Scientific). Protein concentration was determined by Bradford's assay using bovine serum albumin as a standard (Bradford, 1976). Measurement of the absorbance at 280 nm was used for the determination of the concentration of the purified recombinant xylanase.

Immobilization of recombinant xylanase and pectate lyase

Three supporting materials were used for immobilization: calcium alginate, Sepabeads EC-EP/M and Sepabeads EC-HA/M. 0.127 mg of xylanase or 0.565 mg of pectate lyase were used for each immobilization. His-tagged enzymes were used for immobilization.

Immobilization on calcium alginate was performed according to Roy et al. (2004) with some modifications. Namely, sodium alginate was dissolved in 50 mM Tris-HCl (pH 9/60°C) for the immobilization of pectate lyase, and in 100 mM Tris-HCl buffer (pH 7/60°C) for the immobilization of xylanase. Homogeneous solutions of alginate and enzyme were dropped in a 0.2 M $CaCl_2$ solution. Calcium alginate beads were stored in this solution for 1 h and then transferred to the buffers mentioned above containing 0.006 M $CaCl_2$.

Immobilization on Sepabeads EC-EP/M was performed according to Pessela et al. (2003) with some modifications. Namely, 50 mM Tris-HCl (pH 9/60°C) was used for pectate lyase immobilization, and 100 mM Tris-HCl buffer (pH 7/60°C) was used for the immobilization of xylanase.

Immobilization on Sepabeads EC-HA/M was performed according to Nawani et al. (2006).

Extraction of pectin and xylan from raw material

Apple pomace and distillers grains were obtained from the local manufacturers of fruit juices and corn ethanol and dried at 60°C. Wheat straw and wheat bran were obtained from a local farmer.

Dried apple pomace was swelled in sodium citrate buffer (pH 4.5/20°C) and treated with laccase (from *Trametes versicolor*; Sigma-Aldrich) in a concentration of 10 U/100 ml for 16 h (Parmar et al., 2013). The treated pomace was collected by centrifugation (10000xg, 10 min) and washed thrice with 50 mM Tris-HCl (pH 7/60°C).

The straw, bran and dried distillers grains were ground using a coffee mill prior to use in experiments to ensure a representative sample. 10 g of the ground material were suspended in 100 ml of water. Extraction of xylan was performed according to Thomsen et al. (2008) with some modifications. Namely, xylan was extracted in three steps: 80°C for 20 min, 180°C for 15 min, and 195°C for 3 min. The extract was filtered, evaporated and then dissolved in 100 mM Tris-HCl (pH 7/ 60°C).

Pectate lyase and xylanase activity assays

Enzymatic activity was determined by measuring the amount of released reducing sugars by the dinitrosalicylic acid (DNS) method (Miller, 1959).

Pectate lyase activity was assayed using PGA (Alfa Aesar), pectin from apple with degree of esterification of 70-75% (Sigma-Aldrich) and pectin from apple pomace as substrates. 9.45 μg of the purified recombinant pectate lyase was used for each reaction. PGA and pectin from the apple assay mixture (215 μl) consisted of 50 mM Tris-HCl buffer (pH 7/60°C) respectively, 1.4% (w/v) substrate and enzyme. Pectin from the pomace assay mixture (500 μl) consisted of 50 mM Tris-HCl buffer (pH 7/60°C), 0.36% (w/v) substrate and enzyme. Reaction mixtures were incubated at 60°C for 4 h. One unit of enzyme activity was defined as the amount of the enzyme releasing 1 μmol of monogalacturonic acid equivalent per min under the conditions described above.

Xylanase assay mixture (600 μl) consisted of 100 mM Tris-HCl buffer (pH 7/60°C), containing 1% (w/v) of substrate (beech wood and birch wood xylan, xylan from wheat straw, xylan from bran as well as that from the grains) and 8 μg of the purified recombinant xylanase JK1. The reaction mixture was incubated at 60°C for 30 min when wood xylan was used as the substrate; for 60 min when the grains were used as the substrate; and for 24 h when the straw and bran were used as the substrate. One unit of enzyme activity was defined as the amount of the enzyme releasing 1 μmol of xylose equivalent per min under the conditions described above.

Effect of pH and temperature on recombinant pectate lyase activity

In order to establish the effect of temperature and pH, recombinant pectate lyase assay was carried out at different temperatures (10-90°C) and pH (4-10). The effect of temperature was determined using 50 mM Tris-HCl buffer (pH 7//20°C/40°C/60°C). The effect of pH was tested using 50 mM acetate (pH 4/60°C), 50 mM MES (pH 5-6/60°C), 50 mM Tris-HCl (pH 7–9/60°C) and 50 mM glycine-NaOH (pH 10/60°C) buffers. Released reducing sugars were determined by the DNS method described above.

Effect of temperature and pH on the enzyme stability

The thermostability of both free and immobilized recombinant enzymes was investigated at temperatures 50, 60, 70, 80 and 90°C after incubation of the enzyme solutions in absence of a substrate for 60 min. Residual activities were determined by DNS method as described above with the beech wood xylan as the substrate for xylanase, and PGA as the substrate for pectate lyase.

The pH stability of recombinant pectate lyase was investigated at pH 4-9 after incubation of the enzyme solution in absence of the substrate for 60 min at 60°C. The following buffers were used: 50 mM acetate (pH 4.0/60°C), 50 mM MES (pH 5.0-6.0/60°C), and 50 mM Tris-HCl (pH 7.0-9.0/60°C).

Analysis of degradation products

Thin-layer chromatography (TLC) of mono- and oligosaccharides was done on Silica gel 60 F_{254} plates (Merck KGaA). The reaction mixtures were incubated as described above. 5 μl of each pectate lyase reaction mixture and 6 μl of each xylanase reaction mixture were applied to the plate.

Pectic oligosaccharides were separated with the *n*-butanol-water-acetic acid (5:3:2, vol/vol/vol) solvent system (Soriano et al., 2006). Oligosaccharides were detected by spraying the dried plates with a solution of 3% (w/v) phosphomolybdic acid and 10%

(v/v) sulfuric acid in ethanol, and heating at 120°C for 10 min. Galacturonic, digalacturonic and trigalacturonic acids (Sigma-Aldrich; 10 mg/ml each) were used as size markers.

Xylooligosaccharides were separated with the *n*-propanol-ethanol-water (7:1:2, vol/vol/vol) solvent system (Sunna et al., 2000). Sugars were detected after the plate was dried and dipped in a 5% (v/v) sulfuric acid solution in ethanol and then incubated at 110°C for 10 min. Xylose, xylotriose, xylotetraose and xylopentaose (Megazyme; 10 mg/ml each) were used as size markers.

RESULTS

LC/MS based identification, cloning and expression of pectin degradation associated enzymes

Zymographic analysis of extracellular proteins of the thermophilic enrichment culture, grown on PGA and apple pomace, showed a single zone of activity. Zymogram was aligned with the corresponding band on the equivalent SDS/polyacrylamide gel. The band was excised from the gel without substrate and subjected to LC/MS analysis. LC/MS-based analysis identified thermophilic pectate lyase PL47 (EC 4.2.2.2) from the thermophilic *Bacillus* sp. TS-47 with the sequence coverage 44%. Thirteen different peptides were identified: TELIQALGGNNHTNQYNSVPK, GTIDLNVDDNNQPVGPDFYK, DPHFDFEAYLR, EYDPATWGK, EVEGPLEEAR, IMVYVGSNTSIIGVGK, NVDNVIIR, SLGTYFGRPFQQHDGALDIK, NSSDFITISYNVFTNHDK, VTLIGASDSR, MADSGHLR, NEESMYETGTIVDLPNGR, and YIDLVASYNESNTLQLK.

Based on LC/MS results, namely *Bacillus* sp. TS-47, pectate lyase gene sequence AB045986 was used for the construction of the primers P-1/2/3-PL47-F and P-1/2/3-PL47-R targeting the ends of this sequence. Amplification from the genomic DNA of the enrichment culture resulted in a PCR product ~1300 bp in length. The PCR product was sequenced and analyzed. It was determined that the pectin degradation-associated enzyme gene from our study was identical to that of the pectate lyase of *Bacillus* sp. TS-47.

It is noteworthy that the signal peptide MRRWYTNIFLVVLLACLSIPMQANA was not identified by LC/MS; most likely, it was not accidental as the signal sequence is removed in the course of secretion. Therefore, a forward primer for the cloning was designed to target amino acids (26^{th}-30^{th}) just after the signal peptide. The pectate lyase gene was cloned into pET-28c(+) and expressed in *E. coli* BL21(DE3). Recombinant pectate lyase was purified by two chromatographic separation steps. After purification, >95% pure recombinant pectate lyase was obtained.

Characterization of recombinant pectate lyase

The recombinant pectate lyase was active in the temperature range of 20-80°C (Fig. 1a). Optimum temperature was determined to be 60°C. 17.55% of activity against pectin and 23.19% of that against PGA was retained at 80°C. The tag-free enzyme was negligibly more active than His-tagged (data not shown). The highest activity of recombinant pectate lyase was observed at pH 7 when PGA was used as the substrate and at pH 8 when pectin was used as the substrate (Fig. 1b). The pH range of activity was from 4 to 9 for PGA and from 6 to 9 for pectin.

Both His-tagged and tag-free recombinant pectate lyase were stable in the range of pH 4-9 for 60 min at 60°C (Fig. 1c). The highest stability was detected at pH 6-7. The tag-free lyase was thermostable in the range of 50-90°C for 60 min (Fig. 2a). At 90°C, it retained 92.16% of activity. The His-tagged enzyme completely lost its activity at 90°C. Thus, the tag-free enzyme was more thermostable than the His-tagged one.

Based on these data, further experiments were carried out at pH 7 at 60°C.

Purification of recombinant xylanase JK1

Purification of recombinant xylanase JK1 was

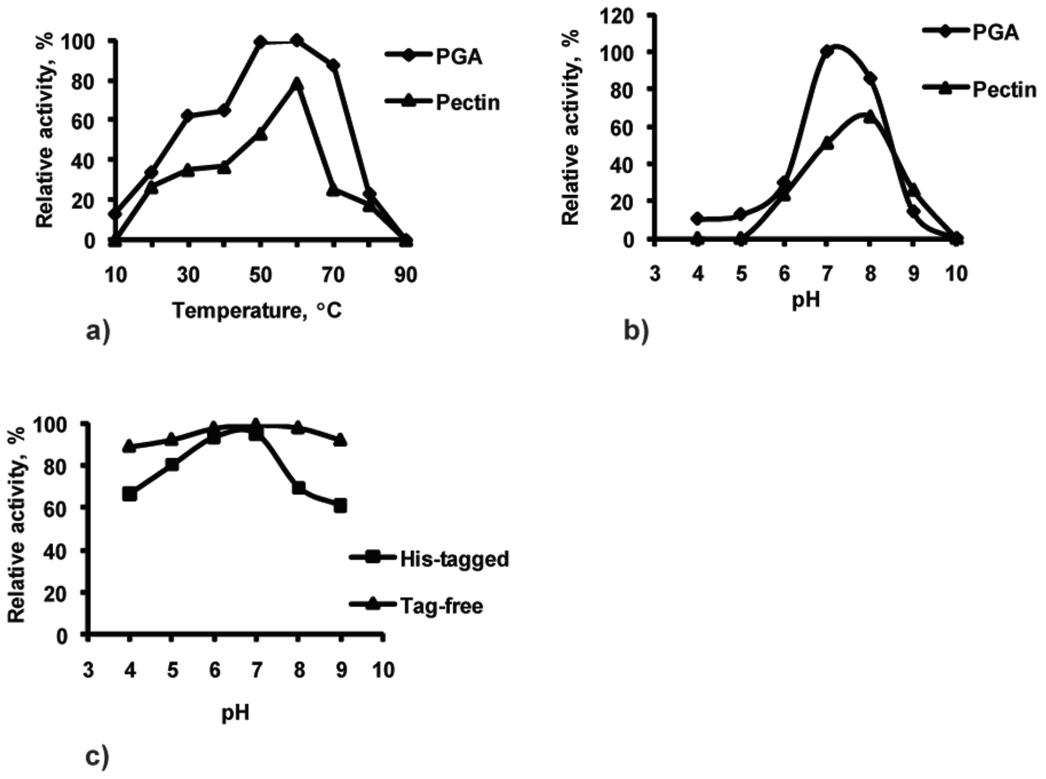

Fig. 1. Effect of temperature on the recombinant pectate lyase activity (a) and that of pH on the recombinant pectate lyase activity (b) and stability (c). a) The recombinant pectate lyase was assayed at various temperatures ranging from 10 to 90°C for 4 h under pH 7 in order to determine the effects of temperature on enzyme activity. b) Effects of pH on the recombinant pectate lyase activity were assayed using different buffers at 60°C for 4 h. c) The pH stability of recombinant pectate lyase was investigated at pH 4-9 after incubation of the enzyme solution in absence of the substrate for 60 min at 60°C. PGA – polygalacturonic acid, Pectin – pectin from apple (Sigma-Aldrich).

scaled-up and modified as compared with the procedure described by Gerasimova et al. (2012). After purification, >99% pure recombinant xylanase JK1 was obtained. The characteristics of the differently purified xylanase JK1 were identical to the previously reported ones (Gerasimova et al., 2012) (data not shown).

Thermostability assays of immobilized recombinant enzymes

For immobilization of pectate lyase and xylanase JK1, three supports were used: calcium alginate, Sepabeads EC-EP/M and Sepabeads EC-HA/M.

Immobilization on EC-HA/M improved the thermostability of lyase at 50°C, but at higher temperatures, immobilization on this support did not enhance the thermostability of the pectate lyase (Fig. 2a). In contrast, immobilization on Sepabeads EC-EP/M increased thermostability in the temperature range of 50-70°C to 108.39-115.56% of activity.

The effect of temperature on the free recombinant xylanase JK1 stability was reported previously by us (Gerasimova et al., 2012). Similar to pectate lyase, immobilization on Sepabeads EC-EP/M increased the thermostability of recombinant xylanase JK1 more than immobilization on EC-HA/M (Fig. 2b). Immobilization on EC-EP/M increased thermostability in the temperature range of 50-80°C to 155-284% of activity, and immobilization on EC-HA/M in the temperature range of 50-70°C to 141-174% of activity. Immobilization on calcium alginate did not

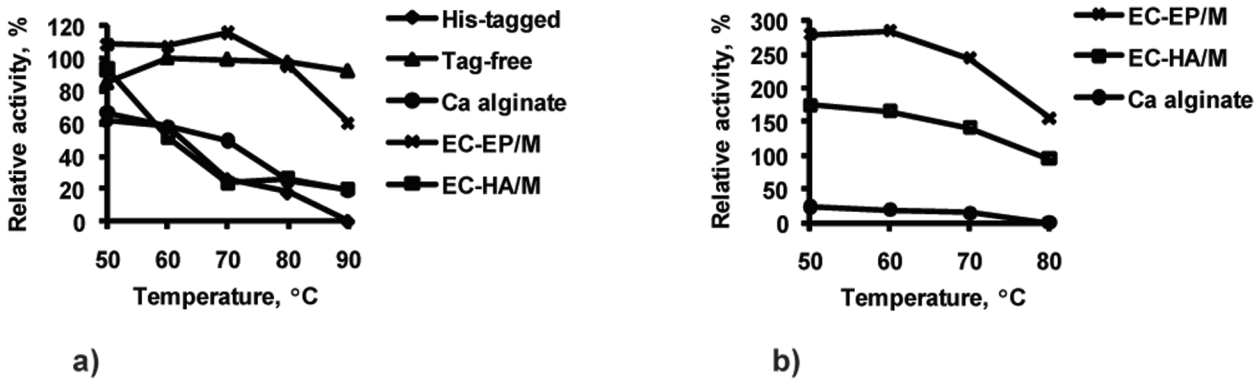

Fig. 2. Effect of temperature on the recombinant enzyme stability. The thermostability of both free and immobilized recombinant enzymes was investigated at temperatures 50, 60, 70, 80 and 90°C after incubation of the enzyme solutions in absence of a substrate for 60 min. a) The thermostability of both free and immobilized recombinant pectate lyase. b) The thermostability of immobilized recombinant xylanase JK1. Ca alginate – thermostability after immobilization on calcium alginate; EC-EP/M – thermostability after immobilization on Sepabeads EC-EP/M; EC-HA/M – thermostability after immobilization on Sepabeads EC-HA/M; His-tagged – thermostability of free His-tagged recombinant pectate lyase; Tag-free – thermostability of tag-free recombinant pectate lyase.

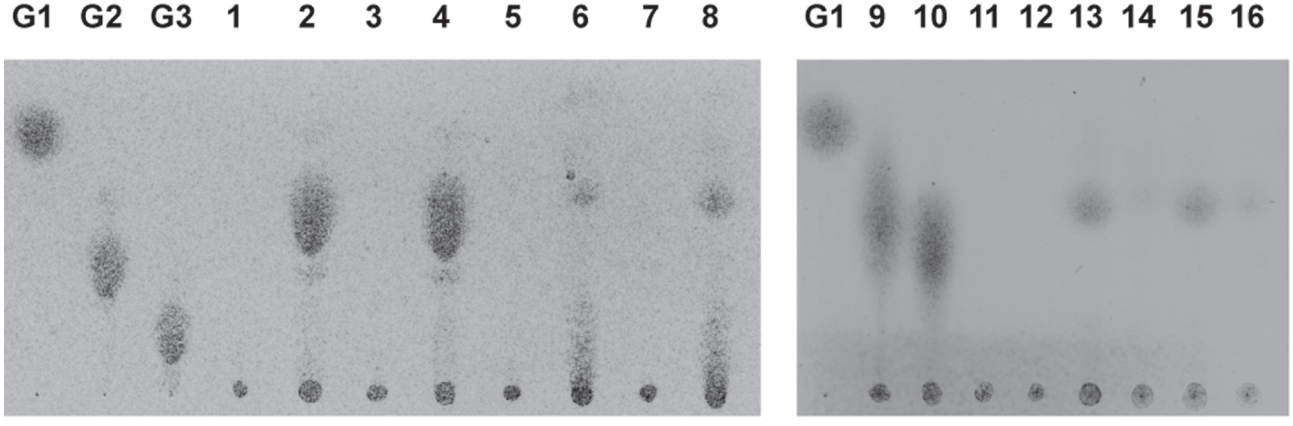

Fig. 3. TLC analysis of pectic oligosaccharides. Lanes: G1-G3 – standards (Sigma-Aldrich); G1 – galacturonic acid; G2 – digalacturonic acid; G3 – trigalacturonic acid; 1, 3, 11 – PGA; 2 – PGA after treatment with the free recombinant pectate lyase at 60°C, pH 7; 4 - PGA after treatment with the free recombinant pectate lyase at 30°C, pH 7; 9 - PGA after treatment with the free recombinant pectate lyase at 50°C, pH 9; 10 - PGA after treatment with the free recombinant pectate lyase at 50°C, pH 6; 5, 7 – pectin from apple; 6 – pectin from apple after treatment with the free recombinant pectate lyase at 60°C, pH 8; 8 - pectin from apple after treatment with the free recombinant pectate lyase at 40°C, pH 9; 12 – pectin from apple pomace; 13 - pectin from apple pomace after treatment with the free recombinant pectate lyase at 60°C, pH 7; 14 - pectin from apple pomace after treatment with the immobilized (calcium alginate) recombinant pectate lyase at 60°C, pH 7; 15 - pectin from apple pomace after treatment with the immobilized (Sepabeads EC-EP/M) recombinant pectate lyase at 60°C, pH 7; 16 - pectin from apple pomace after treatment with the immobilized (Sepabeads EC-HA/M) recombinant pectate lyase at 60°C, pH 7.

enhance the thermostability of either pectate lyase or xylanase.

Activity of recombinant pectate lyase against pectin extracted from apple pomace

Activity assays against PGA and pectin from apple were used as controls in the assays against apple pomace pectin. All three substrates were degraded by the free enzyme. Activity against PGA was higher than that against pectin from apple (Fig. 1ab) and resulted in short oligosaccharides (Fig. 3). Degradation of pomace pectin also showed only short oligosaccharides, while activity against pectin from apple

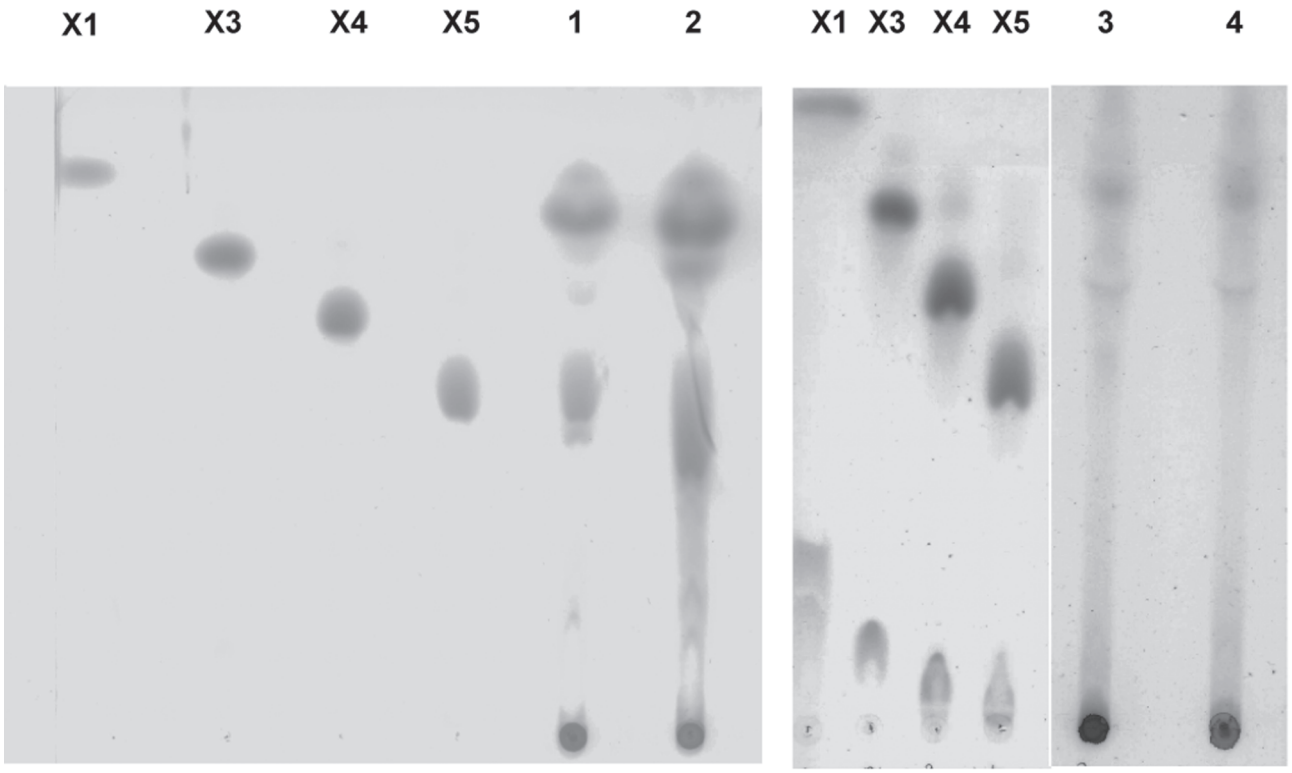

Fig. 4. TLC analysis of xylooligosaccharides. Lanes: X1, X3-X5 – standards (Megazyme), X1 – xylose; X3 – xylotriose; X4 – xylotetraose; X5 – xylopentaose; 1 – birch wood xylan after treatment with the free recombinant xylanase JK1; 2 - beech wood xylan after treatment with the free recombinant xylanase JK1; 3 – xylan from distillers grains after treatment with the free recombinant xylanase JK1; 4 - xylan from distillers grains after treatment with the immobilized (Sepabeads EC-HA/M) recombinant xylanase JK1. All treatments were performed at 60°C, pH 7.

gave additional longer oligosaccharides. Neither changes in pH (7-9) nor those in temperature (30-60°C) affected the length of degradation products. Only the activity assay at pH 6 at 50°C gave longer oligosaccharides (Fig. 3).

It is interesting to note that immobilized pectate lyase did not show activity against pectin from apple but degraded both PGA and pectin from apple pomace (Table 1). Analysis of apple-pomace pectin degradation products showed only short oligosaccharides for both free and immobilized enzymes (Fig. 3). Immobilization on all supports reduced activity of the enzyme (Table 1). The lowest activity was obtained when the pectate lyase was immobilized on calcium alginate. Activity against apple pomace pectin was less affected by immobilization than activity against PGA. Immobilization on Sepabeads EC-EP/M had a negligible effect on the lyase activity against pectin from apple pomace, and this variant of immobilization was the best for pectate lyase in terms of activity.

Activity of recombinant xylanase JK1 against xylan from the straw, bran and distillers grains

Activity assays against birch wood and beech wood xylan were used as controls of JK1 xylanolytic activity (Fig. 4). Although both of them were hydrolyzed, we were unable to hydrolyze xylan from the wheat straw. Both the bran and grains were degraded by the free JK1. The main product of birch wood xylan as well as xylan from the bran and grains degradation by JK1 was disaccharide (Fig. 4; data for the bran not shown). Neither changes in pH (5-9) nor those in temperature (40-80°C) affected the length of the degradation products (data not shown).

Table 1. Activity of immobilized recombinant xylanase and pectate lyase against different substrates. *Relative activity is expressed as a percentage of activity of the respective free enzyme against different substrates that is taken as 100%.

Enzyme	Substrate	Relative activity* (%) after immobilization on		
		Sepabeads EC-EP/M	Sepabeads EC-HA/M	calcium alginate
Pectate lyase				
	polygalacturonic acid	73.56	61.59	55.15
	pectin from apple	0	0	0
	pectin from apple pomace	97.21	89.6	49.45
Xylanase				
	beech wood xylan	17.39	24.75	16.43
	xylan from distillers grains	0	113.14	0
	xylan from wheat bran	85.76	147.47	0

Immobilization remarkably reduced the activity of recombinant xylanase against beech wood xylan (Table 1). Immobilization on calcium alginate eliminated activity of JK1 against the bran and grains. In contrast, immobilization on Sepabeads EC-HA/M remarkably enhanced the activity against both bran and grains. Analysis of the products of hydrolysis of the bran and grains gained by JK1, immobilized on the latter support, matched those obtained by the free enzyme.

DISCUSSION

Plant biomass in the form of cheap agricultural and industrial wastes is abundant throughout the world. Most of these wastes are used as animal feed or burned as an alternative for elimination. Therefore, there is great interest in the reuse of these wastes. Such wastes can potentially be an appropriate starting material for the production of useful value-added compounds for food and healthcare including OS. To our knowledge, thermophilic enzymes, unlike the mesophilic ones, have never been used for this purpose.

For cost-efficient use of enzymes in large-scale industrial applications, high-level expression of enzymes in recombinant hosts is usually a prerequisite (Zhang et al., 2011). Therefore, we examined the potential of recombinant thermophilic enzymes to degrade agricultural wastes: apple pomace, straw, bran and distillers grains. Cloning and expression in *E. coli* BL21(DE3) as well as characterization of the recombinant thermophilic endo-1,4-β-xylanase JK1 was previously reported by us (Gerasimova et al., 2012). In the current work, thermophilic pectate lyase was identified from the thermophilic enrichment culture grown on PGA and apple pomace. Although the identical pectate lyase PL47 was previously reported by Takao et al. (2000), its potential for use in the degradation of cotton fabrics but not for the degradation of apple pomace with the aim to produce POS was evaluated. Takao et al. (2001) expressed pectate lyase PL47 in *Bacillus subtilis* MII12. We chose to clone and express pectate lyase in *E. coli* BL21(DE3), and this shifted the optimal temperature of the recombinant lyase from 70°C (Takao et al., 2001) to 60C. Our His-tagged recombinant enzyme was less thermostable than the tag-free enzyme, which retained even 92.16% of activity after incubation for 60 min at 90°C, and this value is higher than that recorded by Takao et al. (2001). Despite the high thermostability of the tag-free enzyme, the His-tagged enzyme has been chosen for further work because of the simple purification procedure as well as the cost of the removal of the tag by thrombin cleavage. It should be noted that endo-1,4-β-xylanase JK1 was also reported to have quite low thermostability when compared with the respective enzymes (Gerasimova et al., 2012).

Immobilization of enzymes is crucial for the effective utilization and reusability of enzymes in many industrial processes. Enzyme immobilization may increase heat stability and, therefore, improve biocatalyst performance. As both the reusability and in-

creased thermostability were of interest to us, we immobilized pectate lyase and xylanase on three different supports: calcium alginate, Sepabeads EC-EP/M and Sepabeads EC-HA/M. These three supports were not chosen accidentally. Immobilization using calcium alginate has been shown to enhance the thermostability of polysaccharide-degrading enzymes (Roy et al., 2004). Epoxy supports (Sepabeads EC-EP/M in our work) were previously used for one-step purification and immobilization of His-tagged enzymes. Such immobilization on epoxy supports enhanced both the activity and stability of recombinant proteins at high temperatures (Pessela et al., 2003). Hexamethylamino support Sepabeads EC-HA/M was used for the comparison with epoxy support.

In our work, calcium alginate was the worst choice in terms of both thermostability and activity for both immobilized enzymes. In contrast, immobilization on Sepabeads enhanced the thermostability of both recombinant His-tagged enzymes. For xylanase, the highest thermostability was achieved by immobilization on Sepabeads EC-EP/M, but the best activity against bran and grains was obtained by immobilization on Sepabeads EC-HA/M. The straw was not degraded by either the free or immobilized xylanase. Because of the absence of activity against grains of the xylanase immobilized on Sepabeads EC-EP/M, the best choice for immobilization of xylanase could be considered immobilization on Sepabeads EC-HA/M. For pectate lyase, the highest thermostability was achieved by immobilization on Sepabeads EC-EP/M, but activity against apple pomace pectin was slightly reduced by this immobilization. Immobilization on Sepabeads EC-HA/M was worse than that on EC-EP/M in terms of both thermostability and activity. Therefore, immobilization on Sepabeads EC-EP/M could be considered the best choice out of the three supports used in this study for the immobilization of recombinant pectate lyase.

It should be noted that pectin as well as xylan from the industrial wastes could be degraded by both free and immobilized recombinant pectate lyase and xylanase. The only exception was xylan from the straw. The degradation products did not differ for free and immobilized enzymes. Contradictory data concerning the degree of polymerization of effective prebiotics are available in literature (Dongowski et al., 2002; Hernot et al., 2009). In order to determine conditions to monitor the chain length of obtainable OS, we tested the activity of the free enzymes at suboptimal pH and temperature values. The chain length was the same for both optimal and suboptimal assay conditions – short-chain OS were obtained. The only exception was the pectate lyase assay at pH 6 at 50°C, when longer OS were produced.

In conclusion, our results decisively showed that both free and immobilized thermophilic enzymes could be used for the degradation of agro-industrial wastes in order to obtain value-added products – OS.

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