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Characterization of Enzymes Associated with Degradation of Insoluble Fiber of Soybean Curd Residue by *Bacillus subtilis*

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1. Introduction

Soybean curd residue is a residue of soy milk processing in which most soluble nutrients of soybean are extracted to liquid phase, and thus major carbon sources of the residue are insoluble fibers (O'tool, 1999) which amount to 40.2- 43.6 % on a dry matter basis (Van der Riet et al.,1989). Approximately 700,000 tons of the soybean curd residue were produced annually as a byproduct of *tofu* manufacturing in Japan and most of them is incinerated as an industrial waste. We re-utilized the soybean curd residue as a solid substrate of solid-state fermentation (SSF) using *Bacillus subtilis* (Mizumoto et al., 2006).

The insoluble fibers of soybean consist of cellulose, hemicellulose and lignin. Cellulose is the most abundant biological polymer on earth and is the major constituent of the plant cell wall. This lineal polymer is composed of D-glucose subunits linked by β-1,4 glycosidic bonds forming cellobiose molecules and the long chains are linked together by hydrogen bonds and van der Waals forces (Perez et al., 2002). Hemicellulose is a complex of polymeric carbohydrates which contains xylan, xyloglucan, (heteropolymer of D-xylose and D-glucose), glucomannan (heteropolymer of D-glucose and D-mannose), galactoglucomannan (heteropolymer of D-galactose, D-glucose and D-mannose) and arabinogalactan (heteropolymer of D-galactose, D-glucose and arabinose). Among them, xylan, a complex polysaccharide comprising a backbone of xylose residues linked by β-1,4-glycosidic bonds, is the major component. Xylan is the second most abundant polysaccharide in nature, accounting for approximately one-third of all renewable organic carbon on earth (Collins et al., 2005). Lignin is an amorphous non-water soluble and optically inactive heteropolymer. It consists of phenylpropane units joined together by different types of linkages (Perez et al., 2002) Although lignin is the most abundant polymer in wood fiber along with cellulose, its content in non-wood fiber such as straw, grass and seed hull is low (Sun & Cheng, 2002). The lignin content in the soybean seed coat is reported to be low (Krzyzanowski et al., 2001), and thus it is speculated that the soybean curd residue contains relatively small amount of lignin.

B. subtilis has ability to produce several antibiotics with a variety of structures, especially peptides that are either ribosomally or non-ribosomally synthesized (Leclere et al., 2005; Ongena et al., 2005; Stein, 2005). We previously isolated several strains of *B. subtilis* and the

wild strains and their derivatives suppressed 26 types of plant pathogen *in vitro* (Phae et al., 1990) and a fungal disease *in vivo* (Asaka & Shoda, 1996) by producing three lipopeptide antibiotics, iturin A, surfactin and plipastatin (Asaka & Shoda, 1996; Hiraoka et al., 1992; Tsuge et al., 1996, 1999). The suppressive effect of one of the isolates, *B. subtilis* RB14, was mainly associated with the cyclolipopeptide antibiotic iturin A, which contains seven α-amino acids and one β-amino acid. *B. subtilis* RB14-CS, a derivative of the original strain RB14 and a sole producer of iturin A, produced iturin A in SSF using the soybean curd residue 3-fold higher than in submerged fermentation (SmF) (Mizumoto et al., 2006). This suggests that RB14-CS could degrade some kinds of insoluble fibers in soybean curd residue and utilize them as carbon sources during SSF. In this chapter, insoluble fibers in soybean curd residue that RB14-CS could degrade during SSF were clarified and the fiber-degrading enzymes were purified and characterized.

2. Materials and methods

2.1 Strain
B. subtilis RB14-CS which is a spontaneous mutant derived from RB14-C is a single iturin A producer. *B. subtilis* RB14-C is a streptomycin-resistant mutant from a parent strain RB14 and is a co-producer of the antibiotics iturin A and surfactin (Asaka & Shoda, 1996).

2.2 Solid-state fermentation (SSF)
The detail of SSF was described in the previous paper (Mizumoto et al., 2006). The L medium used for the growth of the bacterium contained 10 g of Polypepton (Nippon Pharmaceutical Co., Tokyo, Japan), 5 g of yeast extract and 5 g of NaCl (per liter). One ml of L medium culture broth after 24 h cultivation at 30°C was inoculated into 100 ml of number 3S (no. 3S) medium consisting of 30 g of Polypepton S (Nippon Pharmaceutical Co., Tokyo), 10 g of glucose, 1 g of KH_2PO_4, and 0.5 g of $MgSO_4 \cdot 7H_2O$ (per liter) (pH 6.8), and the culture was incubated at 120 strokes per minute (spm) at 30°C for 24 h in a shaking flask and used as a seed for SSF.

The soybean curd residue was supplied from a *tofu* company in Tokyo and stored at - 20°C. Each of fifteen grams of thawed soybean curd residue was placed in a 100-ml conical flask and autoclaved twice at 120°C for 20 min at an interval of 8-12 h to kill spore-forming microorganisms inhabiting the material. After cooling to room temperature, the following solutions were added as nutrient supplements for every 15 g of soybean curd residue and moisture content was adjusted to 79%: 833 μL of 0.45 g glucose /ml, 75 μL of 1 M KH_2PO_4, 150 μL of 1 M $MgSO_4 \cdot 7H_2O$ and 367 μL of deionized distilled water. Then, 3 mL of an RB14-CS culture grown in no. 3S medium was added to 15 g of soybean curd residue and mixed with a stainless steel spatula. All flasks were incubated statically in a water incubator at 25°C, and at a specified time, one flask was taken and the whole soybean curd residue in a flask was used as a sample for analysis.

2.3 Preparation of samples for acid and neutral detergent fiber analysis
After 5 days of SSF by *B. subtilis* RB14-CS, the whole solid culture was dried by microwave and ground by using a pestle and a mortar. Raw soybean curd residue was used as a control.

2.4 Acid and neutral detergent fiber analysis
2.4.1 Acid detergent fiber
The content of acid detergent fiber, which contains mainly cellulose and lignin, was analyzed in the following manner (Van Soest, 1963). In a 150 mL-flat bottom flask, 0.45 – 0.55 g of ground sample was weighed using micro-balance and 50 mL of acid detergent solution (20 g/L cetyl trimethylammonium bromide in 0.5 M sulfuric acid) was mixed. The flask was placed in an oil bath under the cold water condenser and boiled within 5-10 min. Sample was refluxed for 60 min from onset of boil. After approximately 30 min, the inside of flask was washed with minimal amount of acid detergent solution. After refluxed, sample was filtrated under reduced pressure with a tared Gooch crucible. The crucible was washed twice with hot water, then twice with acetone and was dried at 105°C overnight. After cooled to room temperature in a desiccator, the weight of the crucible was measured.

2.4.2 Neutral detergent fiber
The content of neutral detergent fiber which contained mainly cellulose, lignin, and hemicellulose was analyzed in the following manner (Van Soest, et al., 1991). In a 300 mL-round bottom flask, 0.45 – 0.55 g of ground sample, 50 mL of neutral detergent solution (13.5 g of sodium dodecyl sulfate, 8.38 g of EDTA disodium salt, 3.07 g of $NaB_4O_7 \cdot 10H_2O$, 5.18 g of $Na_2HPO_4 \cdot 12H_2O$ and 4.5 mL of tryethylene glycol per 450 mL) and 0.5 g of sodium sulfite were mixed. The flask was placed in an oil bath under the cold water condenser and boiled for 5 min. After 5 min of boiling, 2 mL of α-amylase solution, which consists of heat-stable α-amylase (Kleistase T10S; Daiwa Kasei, Shiga, Japan) and 50 mM sodium phosphate buffer (pH 6.0) (1:39 [vol/vol]), were mixed. Then, the sample was refluxed for 60 min. After approximately 30 min, the inside of flask was washed down with minimal amount of neutral detergent solution. After refluxed, the sample was filtrated under reduced pressure with a tared Gooch crucible. The crucible was filled with 2 mL of α-amylase solution and hot water, and incubated for at least 2 min. Then, the crucible was washed twice with hot water, and then twice with acetone. The crucible was dried at 105°C overnight. After cooled to room temperature in a desiccator, the weight of the crucible was measured.

2.4.3 Calculation of content of insoluble fibers
As the amount of acid detergent fiber was regarded as total amount of cellulose and lignin, the amount of the neutral detergent fiber minus the amount of acid detergent fiber was regarded as the content of hemicellulose.

2.5 Iturin A production in liquid culture using insoluble fibers
In a 200-mL conical flask, 40 mL of liquid medium consisting of 10 g of fibrous carbon sources, 10 g of Polypepton S, 1 g of KH_2PO_4 and 0.5 g of $MgSO_4 \cdot 7H_2O$ (per liter) (pH 6.8) was prepared. As fibrous carbon sources, xylan (Tokyo Chemical Industry, Tokyo, Japan), avicel, carboxymethyl cellulose, and pectin were used. As a control carbon source, glucose was used. Four hundreds μL of a seeding culture was inoculated into the medium and the flasks were incubated at 30°C at 120 spm.

For measurement of iturin A concentration, 1 mL of culture broth was acidified to pH 2.0 with 12 N HCl. Iturin A was collected by centrifugation at 18,000 ×g, at 4°C for 10 min, and extracted with 1 mL of methanol. The extract was injected into a high-performance liquid chromatography (HPLC) with a column (Chromolith Performance RP-18eb 4.6 mm

diameter× 100 mm height, Merck, Germany) to determine iturin A concentrations. The HPCL system was operated at a flow rate of 2.0 mL/min with acetonitrile-10 mM ammonium acetate (65:35 [vol/vol]) at a column temperature of 40°C. The elution was monitored at 205 nm by a UV detector (880-UV, Intelligent UV/VIS Detector, Jasco, Tokyo, Japan).

Although iturin A has 8 homologues with different side-chain structures (Asaka & Shoda, 1996), the concentration of iturin A was defined as the total amount of five major homologues. The correlation between the peak heights and the concentration of pure iturin A (Sigma-Aldrich, Tokyo, Japan) was used for quantification. Iturin A concentration was expressed as µg/ g initial wet soybean curd residue.

2.6 Xylanase activity assay

Dinitrosalicylic acid (DNS) solution was prepared in the following manner. Solution A was prepared by mixing 300 mL of 4.5 % NaOH, 880 mL of 1 % 3,5- DNS and 225 g of potassium sodium (+)-tartrate tetrahydrate. For the preparation of solution B, 22 mL of 10 % NaOH and 10 g of phenol was mixed and filled up to 100 mL. To 69 mL of the mixture, 6.9 g of $NaHCO_3$ was added. Solutions A and B were mixed thoroughly and placed at room temperature for 2 days. After filtration, the mixture was used as DNS solution.

Xylanase activity was determined by measuring the amount of reducing sugar released from xylan. One hundred µL of enzyme sample was added to 1 mL of 1 % xylan in 100 mM sodium phosphate buffer (pH 6.5) in a test tube (15 mmΦ × 10.5 cm) and incubated statically at 50°C for 5 min. Two mL of DNS solution was added and cooled immediately in an ice bath. Then the test tubes were boiled for 5 min and cooled in an ice bath. After centrifugation at 18,000×g at 4°C for 5 min, absorbance of the supernatant at 540 nm was measured by spectrophotometer (UV2400; Shimadzu, Kyoto, Japan). Xylose was used as the standard. One unit (U) of xylanase activity was defined as the amount of enzyme that liberates 1 µmol of reducing sugars (xylose equivalent) per min.

2.7 Measurement of xylanase activity during SSF

SSF was carried out as described in Section 2.2 without addition of glucose. One gram of solid culture sample and 9 ml of sterile distilled water were mixed in a sterile 18-mm-diameter test tube, the test tube was vortexed thoroughly and shaken at 150 spm for 5 min at room temperature. The suspension was centrifuged at 18,000 ×g at 4 °C for 10 min and the supernatant obtained was used for xylanase assay.

2.8 Measurement of concentration of protein

Protein concentrations were determined by the Bradford method (Bradford, 1976) with the Protein Assay Kit II (Bio-Rad, Tokyo, Japan) with bovine serum albumin as the standard protein.

2.9 Purification of xylanase

Solid cultures (90 g) incubated for 5 days in SSF were mixed with 900 mL of distilled water and stirred for 10 min. The suspension was centrifuged at 6,500×g at 4°C for 20 min and the supernatant was frozen at -20°C and then thawed. The sample was centrifuged for removal of polysaccharides under the same condition. Ammonium sulfate was added to the

supernatant to 30 % saturation, and the precipitate was removed by centrifugation. Then, ammonium sulfate was added to 70 % saturation. The precipitate was recovered by centrifugation, suspended in 50 mM MES buffer (pH 6.0) and dialyzed overnight against the same buffer. Then the sample was concentrated by ultrafiltration with YM10 (molecular mass cut-off 10 kDa; Advantec, Tokyo, Japan).

The concentrate was applied to a CM-Toyopearl column (1.3 cm Φ×8.3 cm; Tosoh, Tokyo, Japan) pre-equilibrated with buffer A (50 mM MES buffer, pH 6.0), and fractions were eluted with a continuous linear gradient of 0-0.5 M NaCl in buffer A (total volume 120 mL). The flow speed and the volume of one fraction were 4 mL/min and 8 mL, respectively. In this process, xylanase activity was detected in two fractions, one of which was trapped in the column (Fraction I) and the other was not trapped in the column but passed through (Fraction II). These fractions were subjected to further purification processes.

Fraction I was concentrated using Centriprep YM-10 (molecular mass cut-off 10 kDa; Millipore, Tokyo, Japan), diluted with buffer A and applied to a RESOURCES column (0.6 cm Φ×3.0 cm; Pharmacia Biotech, Uppsala, Sweden) pre-equilibrated with buffer A. Fractions were eluted with a continuous linear gradient of 0-0.15 M NaCl in buffer A (total volume 30 mL). The flow speed and the volume of one fraction were 1 mL/min and 1 mL, respectively. The xylanase active fractions were concentrated with Centriprep YM-10 and applied to a Superdex 75 column (1.6Φ×60 cm; Amersham Bioscience, Tokyo, Japan) pre-equilibrated with buffer A containing 0.2 M NaCl. The elution was carried at a flow rate of 1 mL/min and a volume of one fraction was 2 mL.

The pH of the Fraction II was adjusted to 9.5 by adding NaOH and applied to a QAE-Toyopearl (1.6Φ×3.7 cm; Tosoh) pre-equilibrated with buffer B (25 mM piperazine buffer, pH 9.5), and fractions were eluted with a continuous linear gradient of 0-0.5 M NaCl in buffer B (total volume 120 mL). The flow speed and the volume of one fraction were 4 mL/min and 8 mL, respectively. The xylanase active fractions were concentrated with Centriprep YM-10, and fractions were diluted with buffer B and applied to a QAE column. Step elution was performed with 0.07 M NaCl (total elution volume 96 mL). The flow speed and the volume of one fraction were 4 mL/min and 8 mL, respectively.

The xylanase active fractions were supplied to the subsequent Butyl-Toyopearl chromatography. A column of Butyl-Toyopearl (1.6Φ×4.5 cm; Tosoh) pre-equilibrated with 25 mM piperazine buffer containing 1 M ammonium sulfate was used. Ammonium sulfate was added to the active fractions and its concentration was adjusted to 1 M. This solution was then applied to the column and the elution was carried out with a linear gradient of 1-0 M ammonium sulfate in 25 mM Piperazine buffer (total volume 180 mL). The flow speed and the volume of one fraction were 4.5 mL/min and 9 mL, respectively.

2.10 Molecular mass determination

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 12.5 % gel in accordance with the Laemmli method (Laemmli, 1970). M. W. Marker "Daiichi" II (Daiichi Pure Chemicals, Tokyo, Japan) was used as a molecular mass marker. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 (CBB).

2.11 N-terminal sequence analysis

SDS-PAGE of xylanases was performed according to the above-described method and then the xylanases on the gel were electroblotted to a commercial membrane (Immobilon-P;

Millipore, Tokyo, Japan) with a horizontal blotting apparatus (ATTO, Tokyo, Japan). For the blotting of pure enzyme of Fraction II, 0.01 % of SDS was added to transfer buffer to improve protein transfer efficacy. Parts of the membrane blotted with xylanases were cut out and then amino acid sequencing analysis was performed with an amino acid sequencing apparatus (PPSQ-21; Shimadzu, Kyoto, Japan) according to the standard method (Edman, 1949).

Searches for homologous amino acid sequences were performed by a *B. subtilis* database BSORF (http://bacillus.genome.jp/) and the nonredundant database at The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) with the BLASTP.

2.12 pH and temperature profiles and thermostability of xylanases

Xylanase activity was examined in pH range of 3.0 to 11.0. For pH from 3.0 to 4.0, 100 mM sodium citrate buffer was used. For pH from 4.0 to 6.0, 100 mM sodium acetate buffer was used. For pH from 6.0 to 8.0, 100 mM sodium phosphate buffer was used. For pH from 8.0 to 9.0, 100 mM Tris-HCl buffer was used. For pH from 9.0 to 11.0, 100 mM glycine-NaOH buffer was used. To investigate the effect of temperature, the xylanase activity was measured at 20-70°C at pH 6.5. Xylanase thermostability was measured at 50, 55 and 60°C.

2.13 Thin layer chromatography (TLC) analysis of the digestion products

The digestion products of xylan and xylooligosaccharides (Wako Pure Chemical Industries, Osaka, Japan) by xylanase were analyzed by thin layer chromatography (TLC) according to the method previously reported (Kiyohara et al., 2005) with some modifications.

As a substrate solution, 0.5 % xylan or 0.5 % xylooligosaccharides in 100 mM sodium phosphate buffer (pH 6.5) was used. In a test tube (15 mmΦ × 10.5 cm), 0.5 mL of substrate solution and 0.5 mL of enzyme solution containing 0.5 U of xylanase in 100 mM sodium phosphate buffer (pH 6.5) were mixed and the reaction mixture was incubated at 120 spm at 37°C. After 1, 3, and 16 h of incubation, 100 µL of reaction mixture was sampled to microtube, and mixed with 200 µL of ethanol. Then, the mixture was centrifuged at 18,000×*g* for 10 min and the supernatant obtained was evaporated with a centrifugal concentrator (VC-36N; Taitec, Saitama, Japan). The dried material was dissolved in distilled water and spotted on a Silica Gel 60 TLC plate (Merck, Tokyo, Japan), which was then developed with *n*-butanol/acetic acid/ water (10:5:1, by vol.). After development, the TLC plate was sprayed with aniline hydrogen phthalate reagent. The reagent consisted of 0.93 g of aniline, 1.48 g of phthalic anhydride, 84.5 mL of *n*-butanol and 15.5 mL of distilled water (Partridge, 1949), and heated at 100°C to visualize the digestion products.

3. Results

3.1 Degradation of insoluble fibers in soybean curd residue by *B. subtilis* RB14-CS in SSF

To evaluate the ability of *B. subtilis* RB14-CS to degrade insoluble fibers in soybean curd residue, residual fibers after SSF were analyzed by acid and neutral detergent fiber methods. The same analyses were repeated three times. The average values of three samples are shown in Figure 1. After SSF of RB14-CS, no change in content of cellulose and lignin was observed. On the other hand, the content of hemicellulose decreased to 15 % of initial one, indicating that RB14-CS degraded hemicellulose in soybean curd residue.
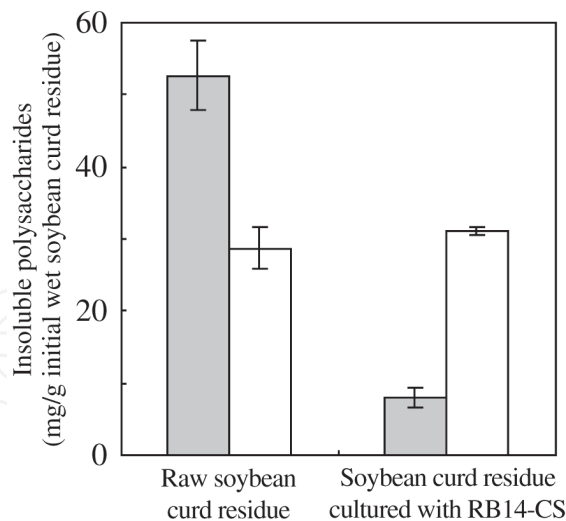
Fig. 1. Analysis of insoluble fiber contents in raw soybean curd residue and soybean curd residue cultured with *B. subtilis* RB14-CS (N=3). Gray bars, hemicellulose; Open bars, cellulose and lignin.

3.2 Iturin A production by *B. subtilis* RB14-CS using insoluble fibers in submerged fermentation

To investigate the effect of insoluble fibers on iturin A production of RB14-CS, each of insoluble fibers was added to a liquid medium as a carbon source and RB14-CS was cultivated in the medium. Results are shown in Figure 2. Xylan exhibited iturin A production at the same level with glucose which has been used as a carbon source for iturin A production in the previous reports (Asaka & Shoda, 1996; Tsuge et al., 2001). Other insoluble fibers, avicel and carboxymethyl cellulose, showed the similar level of iturin A production with control where no additional carbon was added. Pectin, a hardly-soluble or sometimes insoluble fiber which is contained in soybean curd residue (Kasai et al., 2004) did not enhance the iturin A production.
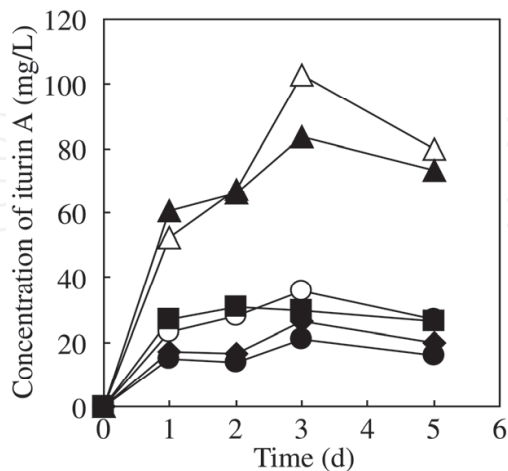

Fig. 2. Iturin A production during submerged fermentation in liquid medium containing fibers (N=3). Symbols: open circles, no additional carbon sources (control 1); open triangles, glucose (control 2); solid circles, pectin; solid triangles, xylan; solid squares, avicel; solid diamonds, carboxymethyl cellulose.

3.3 Xylanase activity of *B. subtilis* RB14-CS during SSF

As RB14-CS degraded xylan, a major hemicellulose in plant cell wall (Beg et al., 2001), in submerged fermentation, xylanase activity was measured during SSF, in which glucose was not added as medium component. Results are shown in Figure 3. The culture of RB14-CS exhibited xylanase activity in SSF. The activity increased after 12 h of incubation, reached the maximum value of approximately 50 U/g wet soybean curd residue at 3 d, and maintained the level during fermentation. When xylanase activity was detected, almost no reducing sugars were detected (data not shown), indicating that RB14-CS immediately utilized the saccharides released from hemicellulose as carbon sources. Changes in cell number and pH were similar to those in SSF of RB14-CS using soybean curd residue previously reported (Mizumoto et al., 2006).
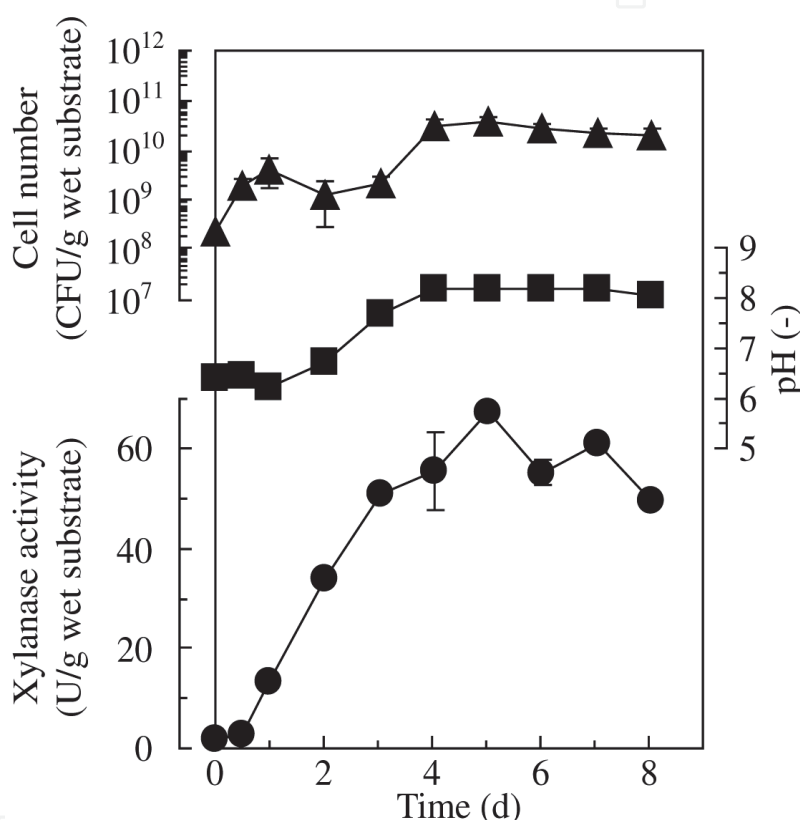

Fig. 3. Xylanase activity of *B. subtilis* RB14-CS during SSF.
Symbols: circles, xylanase activity; squares, pH; triangles, viable cell number.

3.4 Purification of xylanases produced by *B. subtilis* RB14-CS in SSF

Xylanases were purified as described in materials and methods. When the crude enzyme solution was applied to a cation exchange CM-Toyopearl column, xylanase activity was found in both the trapped fraction (Fraction I) and non-trapped fraction (Fraction II). From these fractions, two enzymes were purified and the two enzymes are homogeneous and have different sizes because each single protein band on SDS-PAGE was observed (Figure 4). This indicates that RB14-CS produces two different xylanases. Purified enzymes of Fraction I and II were designated as Xyl-I and Xyl-II, respectively. The molecular masses of the Xyl-I and Xyl-II estimated from SDS-PAGE were 24 and 58 kDa, respectively.
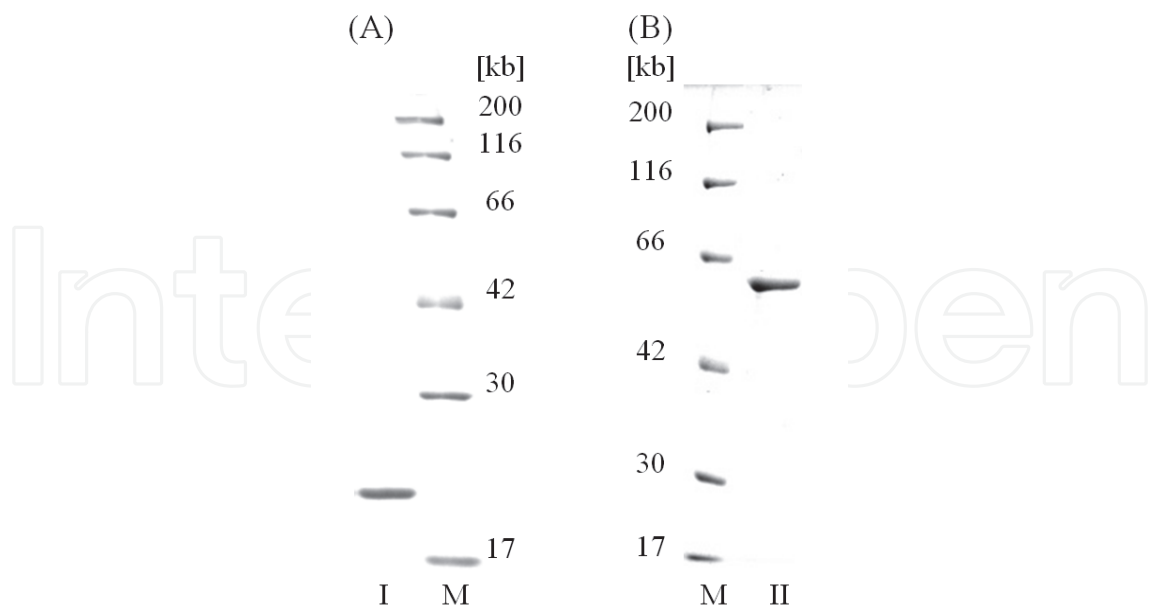
Fig. 4. SDS-PAGE of purified xylanases.(A) Xyl-I, (B) Xyl-II
Lanes: M, molecular mass standards; I, Xyl-I; II, Xyl-II.

3.5 Physicochemical properties of xylanases

Effects of temperature and pH on xylanase activity and thermal stability of the two enzymes are shown in Figure 5. The optimal temperature and optimal pH of Xyl-I were 50-60°C and 6-7, respectively. At 50°C, approximately 30 % of the initial activity of Xyl-I remained after 3 h. At 55 and 60°C, Xyl-I was completely inactivated within 2 and 3 h and the half lives were approximately 18 and 8 min, respectively. The optimal temperature of Xyl-II was 70°C or higher and the optimum pH was 5.5-6. At 50°C, approximately 80 % of the initial activity of Xyl-II remained after 3 h. At 60°C, Xyl-II was inactivated within 3 h and the half life was approximately 40 min.

3.6 Analysis of hydrolytic products

The hydrolysis products released from xylan or xylooligosaccharides by Xyl-I and Xyl-II were analyzed by TLC. From hydrolysis of xylan by both Xyl-I and Xyl-II xylotriose was liberated, but neither xylose nor xylobiose was released. This indicats that these xylanases were not β-D-xylosidase.

3.7 Identification of xylanases by N-terminal sequencing and database matching

The N-terminal sequences of Xyl-I and Xyl-II were determined by automated Edman degradation and compared with databases. Results are summarized in Table 1. Xyl-I displayed 90 % amino acid identity with endo-1,4-β-xylanase (XynA) of *B. subtilis* 168, a standard strain whose complete genome has been sequenced (Kunst et al., 1997). The molecular mass estimated by SDS-PAGE was similar to the database value. Moreover, pI value (9.64) of database was identical to that of purified Xyl-I.

Xyl-II has exactly the same N-terminal sequence as α-amylase (AmyE) secreted by *B. subtilis* X-23 (Ohdan et al., 1999). Actually, Xyl-II exhibited α-amylase activity because reducing sugar was increased when soluble starch was treated with Xyl-II (data not shown). It is
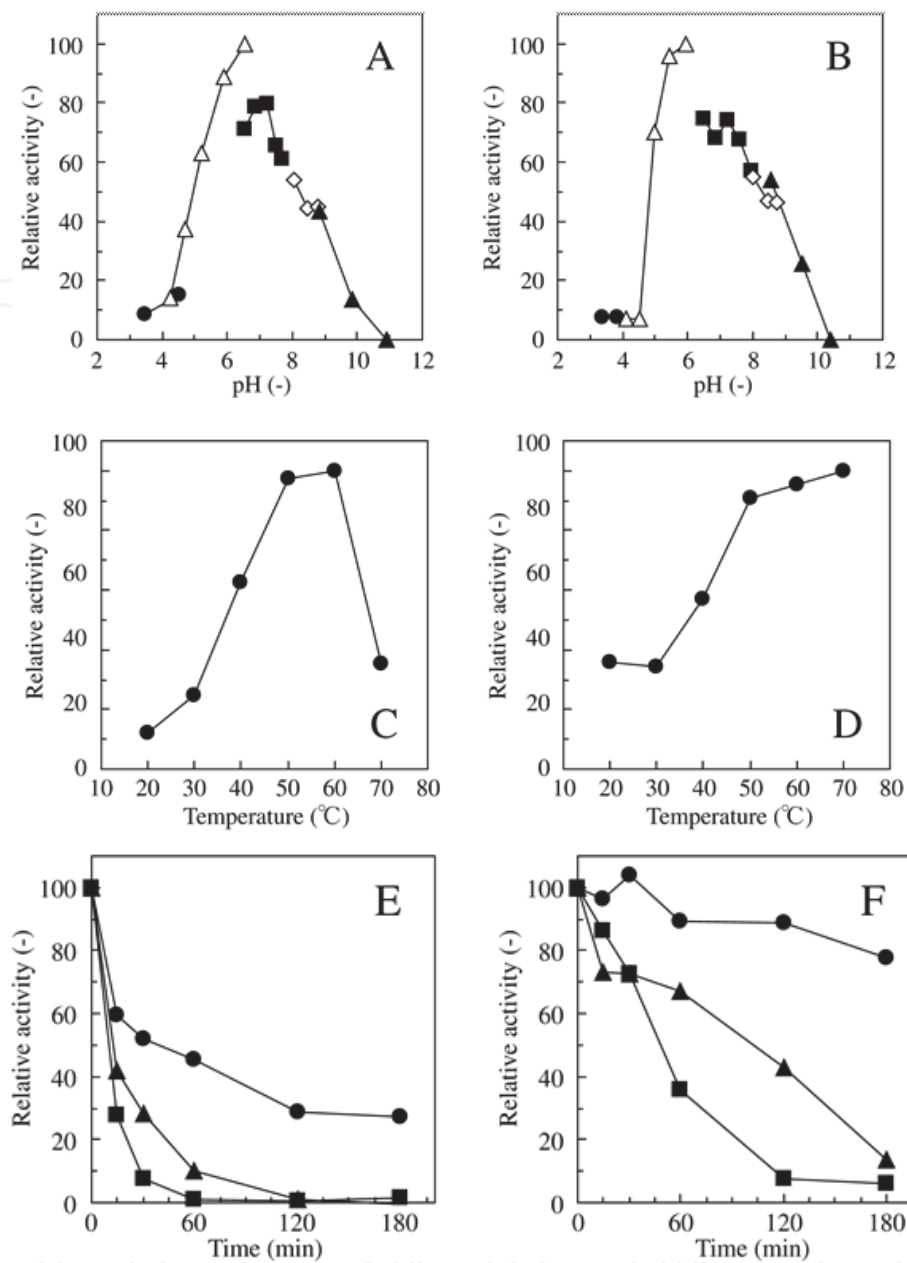
Fig. 5. Effects of pH and temperature on xylanase activities of Xyl-I and Xyl-II. Effects of pH on Xyl-I (A) and -II (B), respectively; Effects of temperature on Xyl-I (C) and -II (D), respectively; Thermal stability of Xyl-I (E) and -II (F), respectively. Symbols in (E) and (F): circles, 50°C; triangles, 55°C; squares, 60°C.

assumed that 45 amino acid residues prior to these sequenced residues deduced from the nucleotide sequence of the *B. subtilis* X-23 are the signal peptide that is removed during the secretion process. Xyl-II also displayed 80 % amino acid identity with α-amylase of *B. subtilis* 168 (Kunst et al., 1997). Although the molecular mass of Xyl-II estimated from SDS-PAGE was different from those in the previous reports, the C-terminal structures of α-amylase of *B. subtilis* were reported to be variable (Ohdan, et al., 1999). The pI value of α-amylase of *B. subtilis* 168 (5.85) was identical with the value of purified Xyl-II. This also reflected in that Xyl-II was trapped in anion exchange chromatography when piperazine buffer of pH 9.5 was used for elution.

This work		Database					
Sequence	Size (kDa)	Sequence	Gene	Protein identity	Size (kDa)	pI	References
Xyl-I AGTDYWQNWT	24	ASTDYWQNWT	*xynA*	endo-1,4-β-xylanase	23	9.64	Kunst et al.
Xyl-II SVKNGTILHA	58	SVKNGTILHA	*amyE*	α-amylase	47, 67	-	Ohdan et al.
		SIKSGTILHA	*amyE*	α-amylase	73	5.85	Kunst et al.

Table 1. N-terminal amino acid sequences of purified xylanases.

4. Discussion

B. subtilis RB14-CS degraded xylan in soybean curd residue and utilized it as a carbon source during SSF by producing xylanases. Xylanases are produced from xylan by fungi, yeast and bacteria, including *Bacillus* sp. (Beg et al., 2001; Blanco et al.,1995; Gallardo et al., 2004;Heck et al., 2005; Sa-Pereira et al., 2003) and physicochemical properties, structures and specific activities of these xylanases were diverse.

In this study, two xylanase-active enzymes were isolated. One of them (Xyl-I)was endo-1,4-β-xylanase (XynA), which has been found in many strains of *Bacillus* sp.(Blanco et al., 1995; Gallardo et al., 2004; Nishomoto et al., 2002). Characteristics of the Xyl-I obtained in this work are similar to those previously reported in that there is β-D-glucosidase activity and the values of optimum pH and temperature of Xyl-I are similar to those in other xylanases (Table 2). Another xylanase-active enzyme obtained (Xyl-II) was identified as α-amylase. As shown in Table 2, physicochemical properties of Xyl-II except for molecular mass were similar to those reported previously. Distribution of α-amylase is wide from common mesophilic bacteria to hyperthermophilic archaeon *Pyrococcus furiosus* (Jorgensen et al., 1997). Alpha-amylase of *B. subtilis* is used commercially in various categories such as starch hydrolysis in starch liquefaction process and additives to detergents for both washing machines and automated dish-washers because of its high thermo-stable activity (Nielsen & Borchert, 2000). As α-amylase, which catalyzes the hydrolysis and transglycosylation at α-1,4- and α-1,6-glycosidic linkages, it doesn't seem to be responsible for degradation of xylan. However, it has been shown that, due to the heterogeneity and structural complexity of xylan, the complete hydrolysis of xylan requires a large variety of cooperatively acting enzymes; such as endo-1,4-β-D-xylanases, β-D-xylosidase, α-L-arabinofuranosidases, α-D-glucuronidases, acetylxylan esterases, ferulic acid esterases and *p*-coumaric acid esterases (Collins et al., 2005). Thus, α-amylase of RB14-CS which hydrolyzed α-1,4- or 1,6-glucoside linkage in the reagent grade xylan used in this study may act as the cooperatively acting enzymes to release reducing sugars from xylan.

Two enzymes isolated in this work liberated xylooligosaccharides but not xylose from xylan. However, almost no reducing sugars were detected when xylanase activity was detected in SSF. This indicates that RB14-CS degraded xylooligosaccharides into xylose and utilized it as a carbon source. RB14-CS may produce other enzymes such as β-D-xylosidase for this reaction.

In recent years, biomass containing hemicellulose, such as agricultural and forestry residues, waste paper, and industrial wastes, has been recognized as inexpensive and abundantly available sources of sugar (Katahira et al., 2004). Since the production of iturin A by RB14-

CS in soybean curd residue was almost equivalent to that when glucose was used as carbon source, the utilization of soybean curd residue will be one possible nutrient in peptide production.

	This work	References	
		Gallardo et al.	Blanco et al.
Molecular mass	24 kDa	24 kDa	32 kDa
Optimum pH	6-7	6	5.5
Optimum temperature	50-60°C	60°C	50°C
Thermal stability	Decreased to 30 % at 50°C after 3 h. Deactivated within 1 h at 60°C.	Remained stable at 50°C for at least 3 h. Deactivated within 1 h at 60°C.	-

(A) Xyl-I

	This work	Reference	(Ohdan et al.)
		Ba-S	Ba-L
Molecular mass	58 kDa	47 kDa	67 kDa
Optimum pH	5.5-6.0	5.5	5.5
Optimum temperature	70°C	65°C	65°C
Thermal stability	80 % was retained after 3 h at 50°C. Deactivated within 2 h at 60°C.	60 % was retained after 10 min at 65°C	30 % was retained after 10 min at 65°C

(B) Xyl-II

Table 2. Comparison of characteristics of purified xylanases with previous reports.

5. Conclusion

Soybean curd residue which is the residue of *Tofu* production was used for nutrients for production of a lipopeptide antibiotic, iturin A in solid state fermentation(SSF) using *Bacillus subtilis*. As the main carbon sources of soybean curd residue were insoluble fiber, we expected that *B. subtilis* produced the soybean curd residue-degrading enzymes. Among insoluble fibers in soybean curd residue, hemicellulose was mainly degraded by *B. subtilis* during SSF. Xylan, a major hemicellulose in plant cell wall was degraded by *B. subtilis*, and two enzymes which showed xylanase activity were purified and identified as endo-1,4-β-xylanase and α-amylase. As productivity of iturin A in soybean curd residue was almost equivalent to that in glucose medium, this study gave a possible way to use soybean curd residue in higher and economical production of lipopeptides.

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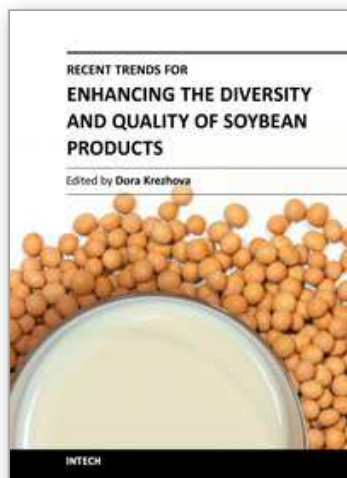
Recent Trends for Enhancing the Diversity and Quality of Soybean Products

Edited by Prof. Dora Krezhova

This book presents new aspects and technologies for the applicability of soybean and soybean products in industry (human food, livestock feed, oil and biodiesel production, textile, medicine) as well as for future uses of some soybean sub-products. The contributions are organized in two sections considering soybean in aspects of food, nutrition and health and modern processing technologies. Each of the sections covers a wide range of topics. The authors are from many countries all over the world and this clearly shows that the soybean research and applications are of global significance.

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