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Gene cloning and characterization of a xylanase from a newly isolated *Bacillus subtilis* strain R5

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A novel mesophilic strain, R5, was isolated from Osaka, Japan. The growth temperature of the strain ranged from 10 to 40 °C with optimal growth at 30 °C. Cells of strain R5 were highly motile small rods. The full-length 16S rRNA sequence was 99% homologous to that of *Bacillus subtilis* strain 168. The optimum pH and NaCl concentration for growth of the strain were 7.0 and 3%, respectively. Based on the biochemical characteristics and 16S rRNA sequences R5 was identified as a strain of *B. subtilis*. The strain R5 produced protease, cellulase, amylase, lipase/esterase, xylanase and a biosurfactant extracellularly. The gene encoding xylanase was cloned and expressed in *Escherichia coli*. The gene encoded a protein consisting of 213 amino acids with a relative molecular mass of 23 kDa. The gene product was purified and examined for enzymatic characteristics. The recombinant enzyme exhibited highest activity at temperatures ranging from 40 to 50 °C and at pH 6.0. The enzyme activity was enhanced in the presence of metal cations. The V_{max} and K_m values of the recombinant enzyme towards xylan from beechwood were 5550 nkat/mg and 4.5 mg/mL, respectively.

[**Key words:** *Bacillus subtilis* R5; 16S rRNA; Extracellular enzymes; Xylanase; Cloning; Kinetic parameters]

Xylan is the major constituent of hemicellulose fraction of plant cell wall matrix. It is a heteropolysaccharide containing substituent groups of acetyl, 4-O-methyl-D-glucuronosyl and α-arabinofuranosyl residues linked to the backbone of β-1,4-xylopyranosyl units. Because of its heterogeneity and complexity, the complete hydrolysis of xylan requires a variety of enzymes such as xylanase, xylosidase, arabinofuranosidase, glucuronidase, acetylxylan esterase, ferulic acid esterase, and *p*-coumaric acid esterase (1–3). All of these enzymes act cooperatively to convert xylan into its constituent sugars. Of them, xylanase (EC 3.2.1.8) is of particular significance. It can catalyze the hydrolysis of β-1,4-xylosidic linkages in xylan to produce small xylo-oligosaccharides and xylose (4). Xylanases have widespread potential applications in textile, waste treatment, animal feed, paper, and biofuel industries (5). Furthermore, they have been used to enhance the digestibility of feed nutrients (6, 7) and for the improvement of wheat flour quality (8).

Xylanases have been grouped into glycosidase families based on the primary structure of the catalytic domains and are normally reported in glycoside hydrolase families 10 and 11 (3). Several xylanase-producing microorganisms have been isolated from a variety of sources (9–12) but still there is a need to explore new microorganisms to find novel xylanases.

The mesophilic strain R5 was isolated from an oily material coagulated in the drains of a restaurant in Osaka, Japan. We have reported that strain R5 produced several extracellular enzymes and involved in the biodegradation of greasy material. The microorganism produced 13,333 nkat of xylanase activity per liter of the growth medium (13). The high enzyme activity indicates the possibility of more than one xylanases excreted by the strain R5. In fact the genome sequence of *Bacillus subtilis* strain 168, the nearest homologue of strain R5, contains six open reading frames encoding various types of xylanases (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=NC_000964). In this paper, we report on the biochemical and molecular characterization of strain R5 as well as cloning and characterization of a xylanase from this microorganism.

MATERIALS AND METHODS

Physiological analysis of isolate R5 Isolate R5 was characterized biochemically by the API 20NE kit (BioMerieux, Marcy l'Etoile, France). All the methods and reagent preparations were carried out according to the instructions of the manufacturer. In addition to the twenty biochemical characteristics given in the API 20NE kit, Gram staining, catalase and oxidase production tests were conducted.

Antibiotic sensitivity In order to determine the sensitivity against antibiotics, strain R5 was cultivated in LB (tryptone 1%, yeast extract 0.5%, NaCl 0.5%; pH7.0) medium containing amoxicillin, ampicillin, chloramphenicol, rifampicin, streptomycin, kanamycin, gentamicin, spectinomycin and tetracycline. All the antibiotics were tested at a final concentration of 100 μg mL^{-1} except for tetracycline, in three independent experiments. Tetracycline was tested at a concentration of 12.5 μg mL^{-1}. Cultures were inoculated in duplicates for all experiments and cells were grown for two days at 30 °C.

General DNA manipulation Restriction enzymes and DNA polymerase were purchased from Fermentas (Maryland, USA) as well as Toyobo (Osaka, Japan). Each enzyme was used according to the instructions of the manufacturer. DNA ligations were performed using DNA ligation kit (Fermentas). Genomic DNA and plasmid DNA were

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isolated by using Fermentas Genomic and Plasmid DNA isolation kits, respectively (Fermentas). A DNA purification kit (Fermentas) was used to recover DNA fragments from agarose gels. Transformations were carried out using calcium chloride method (14).

Cloning of 16S rRNA gene Cloning of partial (500 nt) 16S rRNA gene of strain R5 has been described previously (13). For cloning of the full-length 16S rRNA gene the cloned DNA fragment (0.5 kb) was labeled by DIG DNA labeling and detection kit (Boehringer Mannheim, Mannheim, Germany), and Southern hybridization was performed using chromosomal DNA of strain R5 digested with various restriction enzymes. Both strands of the 16S rRNA gene were determined by an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Database homology searches were performed by using the Basic Local Alignment Search Tool (BLAST) program (15). DNA sequence analyses were performed by using DNASIS software (Hitachi Software, Tokyo, Japan). Multiple-alignment and phylogenetic analyses were performed by using CLUSTAL W program (16) provided by DNA Data Bank of Japan (DDBJ) at their web site (http://clustalw.ddbj.nig.ac.jp/top-e.html).

Cloning and expression of xylanase gene The following set of two oligonucleotide primers were used for amplification of the xylanase gene by polymerase chain reaction (PCR). The forward primer, 5′-GGAGGTAA**CATATG**TTTAAG-3′, was designed on the basis of the N-terminal amino acid sequence of xylanase from *B. subtilis* strain 168. A unique NdeI restriction enzyme site (shown in bold) was present at the start of the gene. The reverse primer, 5′-GCTACCCCTGATTAAGGATG-3′, was designed on the basis of the C-terminal amino acid sequence. The xylanase gene was amplified by PCR that was performed using Taq DNA polymerase (Fermentas) as follows: 3 min at 95 °C; 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C (30 cycles). The PCR amplified DNA fragment was inserted into pTZ57R/T cloning vector (Fermentas). After confirming the sequence, the NdeI–EcoRI restriction fragment was inserted into the pT7-7 expression vector (17) at the corresponding sites. The resulting plasmid, pT-xyl, was used to transform *Escherichia coli* strain BL21(DE3)CodonPlus-RIL. Cells of *E. coli* strain BL21(DE3)CodonPlus-RIL carrying pT-xyl plasmid were grown overnight at 37 °C in LB medium containing ampicillin (50 μg/mL). The culture was inoculated (1%) into fresh LB medium (1 L) containing ampicillin (50 μg/mL) and the cultivation was continued until OD_{660} reached 0.5. Heterologous expression of the xylanase gene was induced by the addition of 0.2 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG) and the incubation was continued for another 4 h at 37 °C.

Purification of recombinant xylanase Cells were harvested by centrifugation at 5000 ×g for 15 min at 4 °C and washed with 40 mM Tris–HCl (pH 8.0). The cell pellet was resuspended in the same buffer, and the cells were disrupted by sonication on ice. Soluble and insoluble fractions were separated by centrifugation (14,000 ×g for 30 min at 4 °C). The proteins in the soluble fraction were precipitated with ammonium sulphate on ice. The precipitates were dissolved in 40 mM Tris–HCl (pH 8.0) and dialyzed against the same buffer. All purification steps were performed at room temperature with commercially available columns (GE Heralthcare, Amersham, Buckinghamshire, UK) unless mentioned otherwise. The dialyzed sample was loaded onto an anion-exchange column (Resource Q) which was equilibrated with 40 mM Tris–HCl buffer pH 8.0. Proteins were eluted with a linear gradient of 0 to 1.0 M sodium chloride in 40 mM Tris–HCl buffer pH 8.0.

Fractions with xylanase activity were pooled and dialyzed against 40 mM Tris–HCl buffer (pH 8.0) containing 1.5 M ammonium sulfate and applied to a hydrophobic column (Resource ISO) equilibrated with 40 mM Tris–HCl buffer (pH 8.0) containing 1.5 M ammonium sulfate. The bound proteins were eluted with a linear gradient of 1.5 to 0 M ammonium sulfate in 40 mM Tris–HCl buffer (pH 8.0).

Protein determination The protein concentration in a solution was determined by Bradford method (18) with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin (BSA) as a standard. The standard curve was plotted using 1 to 15 μg of BSA in 1 mL of Bio-Rad protein assay reagent. The incubations were made at room temperature for 10 min and the absorbance was measured at 595 nm.

Electrophoretic analyses DNA samples were analyzed by 1% agarose gel electrophoresis followed by staining with ethidium bromide and visualizing under short wave ultra violet light. Protein samples were analyzed by polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% sodium dodecyl sulfate (SDS) followed by staining with Coomassie brilliant blue (CBB).

Plate assay *E. coli* strain BL21(DE3)CodonPlus-RIL containing the recombinant plasmid pT-xyl was inoculated on LB agar plate containing 0.2% beech-wood xylan, 50 μg/mL ampicillin and 0.5 mM IPTG. Incubation was made overnight at 37 °C. Following incubation, the plates were stained with 1% Congo-red for 30 min and destained with three washes with 1 M NaCl followed by 0.1 N NaOH. The enzyme activity was examined by a clear zone formation around the colony.

Enzyme assay Xylanase activity was examined by the 3,5-dinitrosalisylic acid (DNS) method (19) by measuring the amount of reducing sugars liberated from beech-wood xylan which was solubilized in 50 mM sodium phosphate buffer at pH 6.0 (20, 21). The reaction mixture was incubated for 10 min at 40 °C. The incubation was followed by the addition of DNS solution. The samples were boiled for 10 min, cooled for colour stabilization, and the absorbance was measured at 540 nm. The xylanase activity was calculated from the calibration curve constructed by using D-xylose as a standard. Two control experiments included in the study were: i) reaction mixture without enzyme and ii) reaction mixture without beech-wood xylan.

Effect of temperature, pH and metal ions on the enzyme activity The optimum temperature of purified recombinant xylanase was determined by incubating the reaction mixture for 10 min at different temperatures ranging from 30 to 70 °C. The pH profile of purified xylanase was evaluated by incubating the reaction mixture for 10 min in the presence of appropriate buffers: 50 mM sodium acetate (pH 4.5–6.0), 50 mM sodium phosphate (pH 6.0–8.0), and 50 mM Tris–HCl (pH 8.0–10.0), at 40 °C. The activity of each sample was then quantified by the assay method as described above.

For examination of the metal ion effect on the enzyme activity, chloride salts of various metals were added in the reaction mixture at a final concentration of 0.1 mM each. Activity was examined at 40 °C and pH 6.0.

Estimation of thermostability Thermostability studies were carried out by incubating the dialyzed protein sample (against 50 mM Tris–HCl pH 7.6) at 50, 60 and 70 °C for various intervals of time and examining the residual activity at 40 °C.

Nucleotide sequence accession numbers The 16S rRNA and xylanase gene sequences reported in this manuscript have been submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession numbers AB257199 and AB457186, respectively.

RESULTS AND DISCUSSION

Identification of isolate R5 The growth temperature of the isolate R5 ranged from 10 to 40 °C. It failed to grow below 10 °C and above 40 °C. Isolate R5 was Gram-positive with a tendency to decolourize easily. It exhibited catalase, oxidase and nitrate reduction activities. It harbored no activities for the tests of urease, arginine dehydrolase, tryptophan deaminase, indole production, and citrate utilization. It could utilize glucose, arabinose, mannose, mannitol and esculin and failed to utilize ribose, xylose, maltose, gluconate and lactose after 48 h incubation at room temperature. These traditional methods were followed by cloning and analysis of complete 16S rRNA gene sequence. When 16S rRNA gene sequence was compared with the sequences from other microorganisms available in the database, it exhibited highest homology of 99% to strains 168 and PY79 of *B. subtilis* (accession numbers AL009126 and EU081774, respectively). The complete 16S rRNA gene sequence was aligned with other available 16S rRNA sequences of various bacteria belonging to genus *Bacillus* and *Geobacillus* and a phylogenetic tree was constructed by the neighbor-joining method (Fig. 1). Strain R5 clustered with strains 168 and PY79 of *B. subtilis*. Based on the 16S rRNA gene sequence homology and the biochemical characterization we identified the isolate as a strain of *Bacillus subtilis*.

Antibiotic resistance The sensitivity/resistance of strain R5 was investigated against various antibiotics. Strain R5 showed no growth in the presence of ampicillin, chloramphenicol, rifampicin, kanamycin, gentamicin and tetracycline indicating that it was sensitive to these antibiotics at concentrations mentioned in the Materials and methods section. It was able to grow in the presence of amoxicillin, spectinomycin and streptomycin showing its ability to resist these antibiotics.

Cloning of xylanase The PCR using sequence specific primers for xylanase gene resulted in the amplification of 0.65 kbp DNA fragment (data not shown). The PCR amplified DNA fragment was ligated in pTZ57R/T cloning vector and *E. coli* DH5α cells were transformed. In order to analyze whether the recombinant plasmid contained the PCR amplified fragment, the recombinant plasmid was digested with EcoRI and HindIII. The digestion resulted in the liberation of 0.65 kbp DNA fragment from the vector indicating that the recombinant plasmid contained the PCR amplified DNA fragment (data not shown). Complete nucleotide sequence of the insert was determined in both the strands and there was no discrepancy in DNA sequence of both the strands. An open reading frame of 642 bp encoding a protein composed of 213 amino acid residues with a calculated molecular mass of 23,354 Da and a pI of 9.44 was identified. The gene sequence was 99% identical to that of *B. subtilis* strain 168 given in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession number M263648 (9). The nucleotides C, G, C, T and C at positions 141, 261, 480, 484, and 498 (M263648 numbering) were replaced by T, A, T, A and T, respectively in strain R5 sequence.