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Research Article

Constitutive High Level Expression of an Endoxylanase Gene from the Newly Isolated *Bacillus subtilis* AQ1 in *Escherichia coli*

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A xylanolytic bacterium was isolated from the sediment of an aquarium. Based on the 16S rDNA sequence as well as morphological and biochemical properties the isolate was identified and denoted as *Bacillus subtilis* (*B. subtilis*) AQ1 strain. An endoxylanase-encoding gene along with its indigenous promoter was PCR amplified and after cloning expressed in *E. coli*. In *E. coli* the recombinant enzyme was found in the extracellular, in the cytoplasmic, and in the periplasmic fraction. The specific activity of the extracellular AQ1 recombinant endoxylanase after 24-hour fermentation was very high, namely, 2173.6 ± 51.4 and 2745.3 ± 11 U/mg in LB and LB-xylan medium, respectively. This activity was clearly exceeding that of the native *B. subtilis* AQ1 endoxylanase and that of 95% homologous recombinant one from *B. subtilis* DB104. The result shows that the original AQ1 endoxylanase promoter and the signal peptide gave a very high constitutive extracellular expression in *E. coli* and hence made the production in *E. coli* feasible.

1. Introduction

1,4-β-Endoxylanase (EC 3.2.1.8) catalyzes the cleavage of the xylan backbone at the 1–4 carbon linkages to produce xylose and xylooligosaccharides. Xylanases have numerous biotechnological applications, both alone and in combination with other enzymes. Second only to cellulose, xylan is one of the most abundant polymers in the world. Along with other xylanolytic enzymes, endoxylanase works synergistically to convert the polymer finally into useful products such as art paper [1], xylose, low-calorie sweetener (xylitol), or bioethanol [2]. Furthermore, xylanases have the potential to reduce the use of environmentally harmful chemicals in the pulp and paper industry and are considered as potential biocatalysts in deinking processes for recycled paper [3]. Additionally, xylooligosaccharides as products of xylan degradation can be used as ingredients in functional food and pharmaceutical products [4].

Xylanases from a panoply of different microorganisms have been described [2, 5], and based on sequence similarities, the enzymes are classified into two glycosyl hydrolases families, that is, family 10 and family 11 [6]. Enzymes belonging to the former family routinely display high molecular masses (more than 40 kDa) whereas those belonging to family 11 have molecular masses of approximately 20 kDa or even below. Family 10 xylanases usually have also detectable cellulolytic activities in addition to their xylanolytic activities whereas members of family 11 do not exhibit such activity [7].

Xylanolitic bacteria from Indonesian habitats have been isolated by our laboratory. Cloning and expression of an endoxylanase gene from a local *Bacillus licheniformis* into *E. coli* using an *E. coli* plasmid promoter have previously been conducted [8]. In this paper, we describe the cloning and efficient expression of a family 11 xylanase gene (*xyn11*) governed by its own promoter. The gene originates from a *B.subtilis* xylanolytic strain that was newly isolated and identified. Endoxylanase production of the isolate was compared to recombinant one expressed in E. *coli* and to the homolog of recombinant endoxylanase coming from *B. subtilis* strain DB104.

2. Materials and Methods

2.1. Biochemical Characterization and Carbohydrate Metabolic Profile. The bacterium was isolated as a xylanase producer strain from the sediment of a small aquarium in Serpong/Indonesia. A single colony with a clearing zone on xylan-supplemented LB agar plate was picked and designated as AQ1. Standard microbiological techniques were applied to determine morphological characteristics such as colony and cell shape, Gram and spore staining. Biochemically, isolate AQ1 was checked for catalase activity, urease reaction, nitrate reduction, starch hydrolysis, indole hydrolysis, and gas formation. Carbohydrate source utilization experiments were carried out applying the API 50 CHB system (bioMérieux, Nürtingen, Germany) and analyzed with the APILAB PLUS 3.3.3 software.

2.2. Bacterial Strains, Plasmids, and Media. Chromosomal DNA from isolate AQ1 was extracted as previously described in [8] and served as the basis for PCR amplification of the AQ1 endoxylanase, and 16S rRNA gene. Plasmid maintenance and expression were done in *E. coli* DH5α (genotypes: F^-endA1 hsdR17 (rk^-mk^+), supE44 thi1 recA1 gyrA (Nalr), relA1D (lacZYAargF), U169 ($\varnothing$80lacZDM15)). The plasmid used for cloning and expression was pGEM-T easy (Promega, USA). 1.5% (w/v) agar Luria-Bertani (LB) plate media containing 100 μg/mL (w/v) ampicillin, 50 μg/mL (w/v) 5-bromo-4-chloro-3-indolyl-β-Dgalactopyranoside (X-gal), and 1 mM isopropyl-β-thiogalactopyranoside (IPTG) were used for selection of *E. coli* harbouring recombinant plasmids. DNA cloning, PCR, and plasmid preparation followed standard protocols as in the work of Sambrook and Russel 2001 [10]. LB agar medium containing ampicillin (50 μg/mL) and 0.7% (w/v) oat spelt xylan was used for monitoring xylanase expression.

2.3. 16*S Ribosomal RNA Gene Amplification.* Amplification by PCR with universal primers 16S-27f (5′-GAGTT TGATCCTGGCTCAG-3′) and 16S-1525r (5′-AGAAAGG AGGTGATCCAGCC-3′) for bacterial 16 rRNA gene was conducted using Taq DNA polymerase (NEB, Hitchin, United Kingdom) under the following conditions After initial 5-minute hot start incubation at 94°C, the mixture was introduced to 30 cycles, each cycle including 1 min at 94°C, 35 s at 50°C, and 2 min at 72°C, then 5 min at 72°C for elongation using a thermal cycler (Eppendorf, Germany). The amplified 16S rRNA gene was then purified using High Pure PCR Clean Up Kit (Roche, Germany). The sequencing was performed by ABI 3100 DNA Sequencer. The primers used for sequencing were as follows: 16S-27f, 16S-343r (5′-CTGCTGCCTCCCGTA-3′), 16S-357f (5′-TACGGGAGGCAGCAG-3′), 16S-519r (5′-G(T/A)ATTACCGCGGC(T/G)GCTG-3′), 16S-536f (5′-CAGC(C/A)GCCGCGGTAAT(T/A)C-5′), 16S-803f (ATTA GATACCTGGTAG-3′), 16S-907r (5′-CCGTCAATTCATTT AGTTT-3′), 16S-114f (5′-GCAACGAGCGCAACCC-3′), 16S-1385r (5′-CGGTGTGT(A/G)CAAGGCCC-3′), and 16S-1525r (5′-AGAAAGGAGGTGATCCAGCC-3′). The DNA sequences were analyzed using Clone Manager Software (Sci-Ed Software, North Carolina, USA).

2.4. Xylanase Gene Amplification, Cloning, and Sequencing. The primers for the amplification of xylanase gene (*xyn*) and its promoter were designed based on the *B. subtilis* strain 168 genome sequence (http://genolist.pasteur.fr/SubtiList/): (5′-GGGGTACCTAGCGTTATTATACTGAAGGGGACGATC-3′) served as a forward primer and (5′-GAAGATCTTTAC CACACACTGTTACGTTAGAACTTCC-3′) as the reverse primer with the underlined nucleotides showing the restriction enzyme site of underlined ones being *Kpn*I and *Bgl*II, respectively. PCR amplification with Taq polymerase using the above primers and the extracted chromosomal DNA of isolate AQ1 as the template was carried out under the following conditions: initial 94°C for 3 min for hot start, then 30 cycles of 94°C 45 s, 52°C 1 min, and 72°C 2 min for each cycle. The PCR product with the predicted size then was extracted and purified from the agarose gel with the Gel extraction Kit (Gene Aid, Taipei County, Taiwan), and after ligating it into the TA cloning vector pGEM T-easy the recombinant plasmid was transformed into *E.coli* DH5 α. The confirmed recombinant pGEM T-easy plasmid was cut by *Eco*RI and the *xyn* gene was removed. The remained vector then religated and transformed into *E. coli* DH5 α,and this clone was used as a negative control. The *xyn* gene from *B. subtilis* DB104, that is, a derivative of *B. subtilis* strain 168 (http://www.uniprot.org/taxonomy/1423#straintable) was also cloned and expressed in the exactly same procedure. For comparison, the AQ1 endoxylanase gene lacking its indigenous promoter was also cloned into pGEM T-easy with the forward primer being 5′-AATGCGGCCGCAA TGTT-TAAGTTTA AAAAGAATTTCT-3′ and the reverse primer 5′-GCTCTAGATTACCACACCACTGTTACGTTAGAACTT-3′ then transformed into *E. coli* DH5 α. The recombinant *E. coli* containing this recombinant plasmid was used for xylanase activity assay in the absence of native putative promoter in the gene. Then from this plasmid the ORF was removed and subcloned behind the T7 promoter in the pBAD/IIIg vector in *E.coli* Top 10 as previously described between sites *Not* I and *EcoR* I [8]. That's done used for determining the molecular mass. For obtaining optimal expression of the gene, induction was done by adding arabinose (0%–0.2%) as suggested in the plasmid vector manual. Sequencing of endoxylanase gene was done using primers matching vector sequences. The DNA sequence was analyzed by performing BLAST searches (http://www.ncbi.nlm.nih.gov/).

2.5. Analysis for 16S rDNA and Endoxylanase Gene. The phylogenetic tree data were obtained by alignment of the different 16S rDNA sequences through the http://align.genome.jp/ server database using the program CLUSTAL W version 1.8 [11] and Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi)) with the standard parameters. The alignments were visually corrected when necessary by GeneDoc Software. Phylograms were obtained

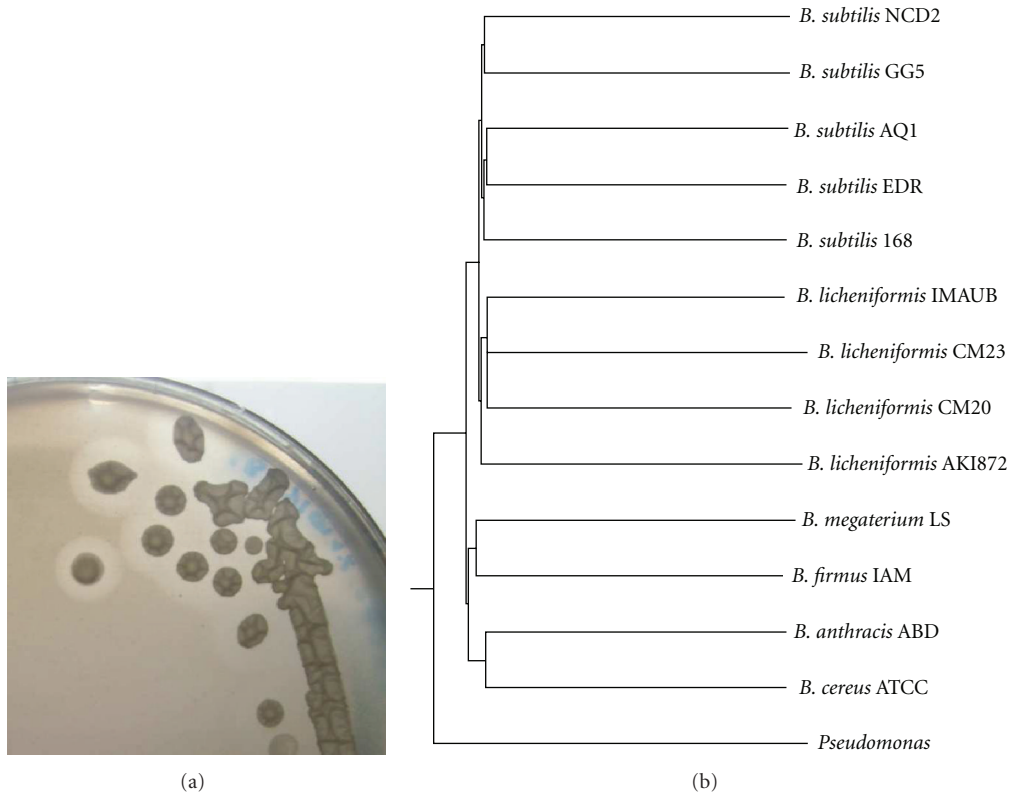


Figure 1: The morphology of newly isolated strain AQ1. Colony morphology on LB xylan agar medium (a) and its phylogenetic tree analyses (b).

by the neighbor-joining method [11]. The following endoxylanase genes used in multiply alignment analyses were retrieved from GenBank: Bfirm: from *Bacillus firmus* (AA Q83579); Paenibac: from *PaeniBacillus* sp (DQ869568); Apunctata: from *Aeromonas Punctata* (D32065).

GenBank Accession Number; The sequences of the 16S rDNA and the endoxylanase gene from this strain AQ1 were then submitted to Genbank.

2.6. Molecular Mass Determinations and Electrophoresis Analysis. The apparent molecular mass of the recombinant endoxylanase was determined using Arabinose induction of AQ1 xylanase gene cloned in *E.coli* with inducible T7 promoter (vector pBAD/IIIg). The induction using different arabinose concentrations (0%–0.2%) was conducted based on the plasmid vector manufacturer's manual. The molecular mass was determined by 0.1% sodium dodecyl sulfate (SDS) and 10% polyacrylamide gel electrophoresis (PAGE). Standards employed were phosphorylase (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

2.7. Enzyme Preparation from Wild Type Strain. Bacterial strains, *B. subtilis* strain AQ1 and strain DB104, were streaked on LB xylan-agar medium, and a single colony with clear zone was picked and inoculated in 5 mL of each different medium (including LB and LB xylan) and growth overnight (~12 hours until OD_{600} = ~0.9), and 0.625 mL of this inoculum was refreshed in a new medium 25 mL in 125 mL Erlenmeyer flasks at 150 rpm at 37°C for 24 hours (Kuehner Shaker, Switzerland). Subsequently the supernatant was used as sample for the enzyme assays.

2.8. Enzymes from Recombinant E. coli. E. coli DH5α expressing the recombinant xylanases was either cultivated as the same method as above mentioned in 25 mL LB or in LB containing oat spelt xylan at different concentrations: 0.1 and 1%, respectively. Media were supplemented with ampicillin (50 μg/mL) at 37°C and cultivated on a shaker (Kuehner Shaker, Switzerland) at 150 rpm for 16 or 24 hours. For confirmation *E. coli* DH5 α containing an empty circularized pGEM T-easy plasmid and *E. coli* DH5 α containing a recombinant pGEM T-easy containing only ORF of AQ1 xylanase, were cultivated and analyzed in the same way to confirm that the xylanase activity is due to the presence of AQ1 *xyn* with its original promoter in the recombinant plasmid pGEM-T easy.

Extracellular fractions were obtained from the supernatant by centrifugation. The cells were subjected to periplasmic fraction preparation which was obtained based on the method described by Huang et al. 2006 [12] by adding the same volume of chloroform to cells suspended in 6.25 mL of phosphate buffer then incubated at room temperature

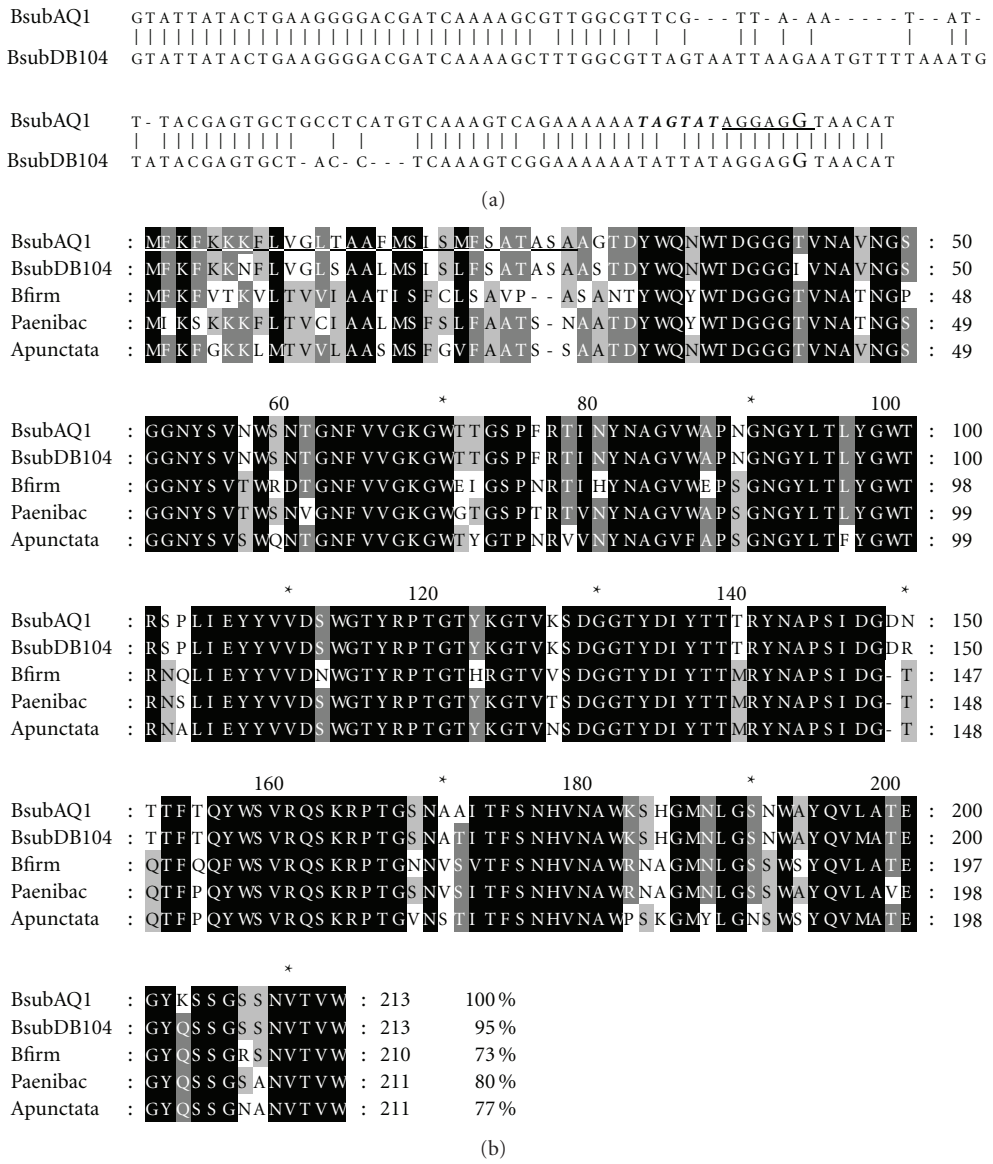


Figure 2: Alignment of AQ1 endoxylanase with other endoxylanases. (a) Comparison of putative promoter region endoxylanase gene of *B. subtilis* strain AQ1 and that of *B.subtilis* strain DB104. Underlined nucleotides correspond to the putative SD site, the large alphabet corresponds to the probable Transcription Start Site (TSS), the bold and italic nucleotides show the probable −10 sequence (b) Alignment of AQ1 endoxylanase's deduced amino acid (this work) with BsubDB104 from *Bacillus subtilis* DB104 (this work) Bfirm from *Bacillus firmus* (AAQ83579); Paenibac from *Paenibacillus* sp. ([9]; DQ869568), and Apunctata from *Aeromonas Punctata* (D32065). In sequence of AQ1 endoxylanase underlined amino acids constitute the putative signal peptide.

for 20 minutes. Then the sample was centrifuged and the aqueous phase containing periplasmic fraction was obtained and used for enzyme assay. The remaining cell pellets from the previous procedure were subjected to cytoplasmic fractions. The cytoplasmic fraction was obtained as follows. The precipitated cells were suspended in 6.25 mL of 20 mM phosphate buffer, pH 7, containing 1 mM mercaptoethanol, then disrupted by sonication (Heat systemXL Ultrasonicator, Japan) at the maximum frequency for 30 s on and 30 s off repeatedly for 5 times at 4°C, and then centrifuged and the supernatant was taken. This cytoplasmic fraction was then subjected to the enzyme assay. As a control for knowing the effect of heat from ultrasonication activity on the total activity, the same amount of culture was sonicated directly, then the supernatant containing all cellular fractions (cytoplasmic, periplasmic, and extracellular) was obtained after centrifugation. This was used as a sample for determining the total activity of all cellular fractions. For *E. coli* DH5 α containing a recombinant pGEM T-easy with DB104 xylanase gene, only the extracellular fraction was taken.

2.9. Assay of Enzyme Activities on Agar Plates. Each 1 μL of the same amount of cell number (OD_{600} = ~0.9) of culture

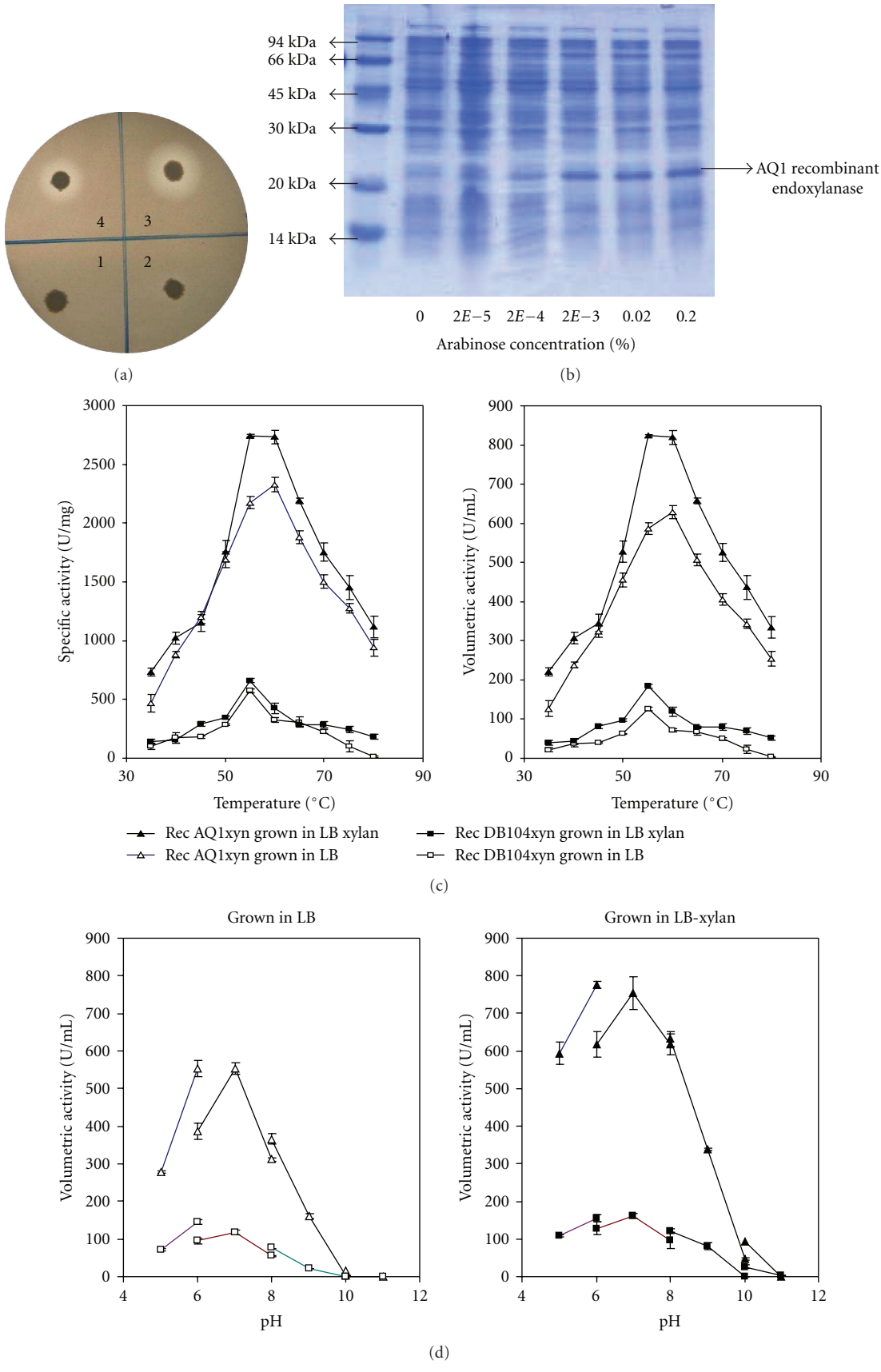
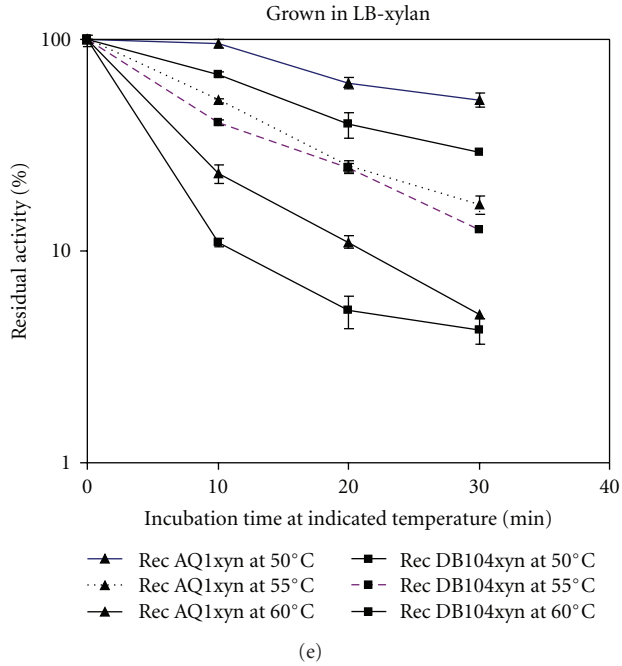


Figure 3: Continued.



(e)

Figure 3: Recombinant *E. coli*s with and without xylanase activity and the comparison of properties of AQ1 and DB104 recombinant xylanase. (a) Recombinant *E. coli*s on LB xylan, recombinant *E.coli* harbouring empty pGEM-T-easy vector (1), recombinant *E.coli* with vector pGEM containing AQ1*xyn* without native promoter (2), recombinant *E.coli* with vector pGEM containing AQ1*xyn* with native promoter (3), and recombinant *E.coli* with vector pGEM containing DB104*xyn* with native promoter (4). (b) The molecular mass of recombinant AQ1 endoxylanase in *E. coli.* For this analysis, the expression of the xylanase gene in cytoplasmic fraction with T7 promoter was used. (c) Effect of temperature on recombinant AQ1 endoxylanase and DB104 endoxylanase grown in LB and LB-xylan medium (triangle was recombinant AQ1 xylanase, black one for that came from *E. coli* grown in LB-xylan, white one for that one grown in LB; square was DB104, black one for that one grown in LB-xylan, white one for that one grown in LB). For this temperature profile, enzymatic activity was measured in 50 mM sodium phosphate pH 7. (d) Effect of pH on recombinant AQ1 endoxylanase and DB104 endoxylanase from *E. coli* grown in LB and LB-xylan medium in different buffers. The reaction pHs were adjusted to 5–11 with the following buffers: 50 mM citrate buffer (pH 5,6), 50 mM phosphate buffer (pH 6–8), 50 mM Tris-HCl (pH 8–10), and 50 mM Tris-Glycine buffer (pH 10,11) (triangle was recombinant AQ1 xylanase, black one for that came from *E. coli* grown in LB-xylan, white one for that one grown in LB; square was recombinant DB104 xylanase, black one for that one grown in LB-xylan, white one for that one grown in LB). (e) Thermostability of recombinant AQ1 endoxylanase and DB104 endoxylanase. Thermostability was determined by preincubating enzyme extract at 50, 55, and 60°C for designated time periods and then assaying the activity in pH 7.0 of 50 mM sodium phosphate at 50°C for 5 min as described in Materials and Methods. The given values in all activity assays are the means of triplicates, and the error bars indicate the standard deviation of these triplicates of independent experiment.

of recombinant *E. coli*s was spotted on LB agar medium containing xylan with ampicillin and incubated at 37°C overnight. Then the clearing zones around the colony were observed.

2.10. Effect of pH and Temperature on Enzyme Activity and Thermostability. The effect of the temperature on extracellular endoxylanase activity was measured in the temperature range of 35–80°C at pH 7 using 50 mM sodium phosphate buffer. The effect of the pH on the activity was measured at 55°C within pH range of 5–11 using 50 mM of the following buffers: citrate buffer (for pH 5, 6), sodium phosphate buffer (for pH 6–8), Tris–HCl buffer (for pH 8–10), and Glysin–NaOH buffer (pH 10-11). Thermostability of the enzyme was measured at 50°C using 50 mM sodium phosphate pH 7 after preincubating in 50, 55, and 60°C for 10, 20, and 30 min. The protein concentration was measured by means of the dye-binding assay method of Bradford and bovine serum albumin (BSA) as the standard protein [13].

2.11. Enzyme Assay. Xylanase activity was measured (each sample in triplicates) based on the Miller method using dinitrosalicylic acid to quantify reducing sugar, D-xylose was used as the standard [14, 15]. 50 μl crude extract at appropriate dilutions in phosphate buffer was mixed with 450 μl of 1% oat spelt xylan in 50 mM of buffer at indicated pH. The mixture was then incubated at the indicated temperature for 5 min; subsequently, 750 μl of DNS reagent (1% dinitrosalicylic acid, 0.2% phenol, 0.05% sodium sulfite, and 1% sodium hydroxide, 20% (w/v) potassium sodium tartrate) was added, to stop the reaction, boiled at 100°C for 5 min, and kept at room temperature; then 250 μl of water was added and the mixture was centrifuged to obtain a clear supernatant. For each sample, the absorbance of sample

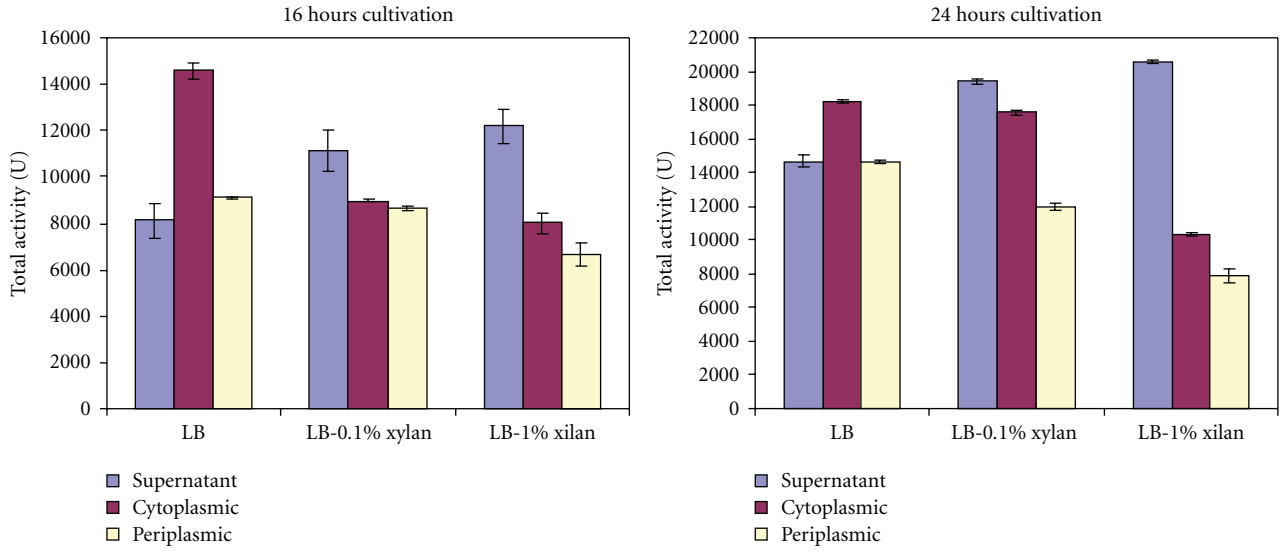


Figure 4: Recombinant AQ1 endoxylanase distribution in cellular compartment of *E. coli* after 16 and 24 hours of cultivation at 37°C of 25 mL medium culture at shaking erlenmeyer flask. The given values in all activity assays are the means of triplicates, and the error bars indicate the standard deviation of these triplicates of independent experiment.

was measured against an avoided reagent at the indicated pH and temperature consisted of the same mixture as in the above sample; however, enzymes were added following addition of DNS into the reaction mixture. The absorbance was measured at 540 nm. The activities of the periplasmic fraction and the supernatant of recombinant *E. coli* were measured by the same procedure. One unit xylanase activity was defined as the amount of enzyme that releases 1 μmol of xylose per min under the assay conditions.

3. Results

3.1. Biochemical Characterization and Carbohydrate-Metabolism Profile of the Isolated Strain. Morphological and biochemical characterization of the xylanase-producing bacterial isolate from the aquarium sediment revealed that the cells were rod shaped, motile, endospore forming, and Gram positive; they reacted positive in catalase, nitrate reduction, urease, and starch hydrolysis but negative in indole production and H_2S formation (Table 1).

The isolate termed AQ1 grew well at temperatures ranging from 25 to 55°C; it formed smooth, convex colonies, presumably due to slime formation which was enhanced at temperatures above 30°C. According to Bergey's manual of determinative bacteriology [16] it belongs to the genus *Bacillus*.

Extended physiological and biochemical characterizations performed by making use of the API 50 CHB kit system [17] revealed that two properties differed from the type strain *B. subtilis* as described in the API manual (N acetyl glucosamine and D-Turanose assimilation, Table 1). Anyway, the APILAB software suggested *B. subtilis* as the species (with identification level of very good).

3.2. Molecular Identification. To prove and to further confirm the identity of the strain to the species level, the entire 16S rRNA sequence was determined (GenBank accession number FJ644629). When compared to other 16S rRNA sequences the strain displayed 99% identity with *B. subtilis* EDR4 as depicted in the phylogenetic tree analysis shown in Figure 1(b). Thus, there is convincing morphological and molecular evidence that isolate AQ1 belongs to the species *B. subtilis*.

3.3. Xylanase Gene Amplification, Cloning, and Sequencing. As outlined in Materials and Methods, a gene of strain AQ1 coding for a family 11 xylanase (*xyn*) including its indigenous promoter was amplified by PCR, cloned, and sequenced. For the primer designing, we predicted the region that might contain a native promoter based on the *B. subtilis* strain 168 using promoter prediction tools (available at http://www.fruitfly.org/seq_tools/promoter.html) and found that 110 bp of upstream region in this *Bacillus* may have a putative promoter with possibility level of 90%. Using the designed primers based on this sequence, we succeeded in cloning and isolating the AQ1 endoxylanase gene. The DNA sequence comprises a 101 bp putative promoter region and the 642 bp spanning ORF starting with the standard initiation codon and ending with a stop codon. The obtained sequence has been submitted to GenBank (accession number FJ644630). The Shine-Dalgarno (SD) sequence strictly matches with the consensus; however, the putative sequence that is strictly similar to −10 and −35 sequence could not be found (Figure 2(a)). This made denomination of a promoter of AQ1 xylanase gene by making use of the same prediction program stay only at the 50% level.

Table 1: Physiological and biochemical characteristics of isolates AQ1 based on the API 50 CHB system and other tests described in Materials and Methods. (+) presence; (−) absence.

Properties detected	Isolate AQ1
Colony color	White
Colony shape	Spread
Elevation	Flat
Gram staining	+
Endospore	Central
Growth at 50°C	+
Growth at 7% NaCl containing medium	+
Growth in anaerobic agar	+
Cell shape	Rod
Hydrolysis of indole	−
Methyl Red	−
Voges-Proskauer	+
Citrate	+
Catalase	+
Nitrate reduction	+
Slime formation	+
Carbohydrates metabolism:	
Glycerol	+
Erythritol	−
D-arabinose	−
L-arabinose	+
Ribose	+
D-xylose	+
L-xylose	−
Adonitol	−
beta-methyl-D-xyloside	−
Galactose	+
Glucose	+
Fructose	+
Mannose	+
Sorbose	−
Rhamnose	−
Dulcitol	−
Galactose	+
Glucose	+
Fructose	+
Mannose	+
Sorbose	−
Rhamnose	−
Dulcitol	−
Inositol	+
Mannitol	+
Sorbitol	+
Alpha-methyl-D-mannoside	−
Alpha-methyl-D-glucoside	+
N-acetyl-glucosamine	−
Amygdalin	+
Arbutin	+
Esculin	+
Salicin	+
Cellobiose	+
Maltose	+
Lactose	+
Melibiose	+
Sucrose	+
Trehalose	+
Inulin	−
Melezitose	−
Raffinose	+
Starch	+
Glycogen	+
Xylitol	−
Gentiobiose	+
D-turanose	−
D-lyxose	−
D-tagatose	−
D-fucose	−
D-arabitol	−
D-arabitol	−
L-arabitol	−
Gluconate	−
2-keto-gluconate	−
5-keto-gluconate	−

The predicted amino acid sequence contains a putative signal peptide as suggested by the corresponding program (http://www.cbs.dtu.dk/services/SignalP/) and shown in Figure 2(b); it comprises the first 28 amino acids at N-terminal.

As mentioned above, primers used for amplification were derived from the genome sequence of *B. subtilis* strain 168. For comparison, the *xyn* gene from a strain 168's derivative, namely, *B. subtilis* DB104 that has exactly the same DNA sequence of endoxylanase gene as that of parent, was also cloned, expressed, and analyzed including its putative native promoter region. The degree of identity of the AQ1 endoxylanase gene including its indigenous promoter was 91% at the nucleotide level, for the predicted protein it was 95% compared to that of *B. subtilis* strain DB104. The promoter region in the upstream region of AQ1 endoxylanase gene was 9 bp shorter than that of DB104 one (Figure 2(a)).

The comparison between AQ1 recombinant endoxylanase and DB104 one showed that these deduced amino acids were 10 different amino acids, 4 amino acids in signal peptide, and 6 amino acids in assumed mature protein, namely at position 7, 13, 16, 21, 30, 43, 150, 171, 197, and 203 (Figure 2(b)).

3.4. Assay of Enzyme Activities on Agar Plates. The clearing zones of colony spot of recombinant *E. coli* with AQ1

endoxylanase (AQ1*xyn*+promoter) were larger than those of the other one with DB104 endoxylanase (DB104*xyn*+promoter) (Figure 3(a)), showing that qualitatively the endoxylanase activity of AQ1 recombinant endoxylanase might be higher than that of DB104. This result was supported by the measurement data of comparison of the activity of these recombinant enzymes, revealing that the productivity of recombinant *E. coli* (AQ1*xyn*+promoter) after 24 hours of cultivation was the highest among those of native endoxylanases resources and another recombinant *E. coli* (DB104*xyn*+promoter) (Table 2). This 24-hour period of cultivation is the optimum time in producing the highest specific activity for native AQ1 and DB104 strain (data not shown).

3.5. Extracellular Endoxylanases Expression in Native Strains and in Recombinant E.coli and Comparison of Properties of the Recombinant Gene Products. The endoxylanase specific activity at 55°C of the wild-type bacterium of *B. subtilis* AQ1 after 24-hour cultivation at 37°C was 306.2 ± 4.2 and 529.7 ± 5.6 U/mg in LB and LB with 1% Oat spelt-xylan, respectively (Table 2). Thus, in the original resource the endoxylanase activity appeared to be constitutively expressed but it was clearly enhanced in medium containing xylan. The same phenomenon also occurred in DB104 endoxylanase resource.

The expression of both AQ1 and DB104 endoxylanase gene from its own promoter in *E. coli* was possible; the recombinant *E. coli* also displayed a constitutive enzyme activity as to be seen from halo formation around the colony on xylan-supplemented LB agar plates incubated overnight at 37°C (Figure 3(a)). The extracellular activity in both LB and LB-xylan medium of AQ1 and DB104 recombinant endoxylanase were measured in different temperature and pH, and compared. We found that, the clearing zone analyses of recombinant *E. coli* were consistent with the comparison of the activity of recombinant extracellular AQ1 endoxylanase and DB104 endoxylanase. Extracellular AQ1 recombinant endoxylanase is always higher than the DB104 one. The volumetric and specific activities of extracellular recombinant AQ1 xylanase were 586.9 ± 13.9 U/mL (2173.6 ± 51.4 U/mg) and 823.6 ± 3.5 U/mL (2745.3 ± 11 U/mg) in LB and LB with1% oat spelt xylan, respectively (Table 2), thus, clearly exceeding enzyme activities of the original strain and also DB104 recombinant endoxylanase. Similar to the phenomenon in the original resource, the recombinant endoxylanase activity in *E. coli* also appeared to be constitutively expressed and it was also enhanced in medium containing xylan.

The comparison of pH and temperature profile and thermostability of AQ1 extracellular recombinant endoxylanase and DB104 one was shown at Figures 3(c), 3(d), and 3(e). The significant difference between AQ1 recombinant endoxylanase and DB104 one is in the higher activity of this AQ1 recombinant endoxylanase—both specific and volumetric activity. The optimal pH and temperature for recombinant endoxylanase AQ1 and DB104 were almost similar, for AQ1 recombinant gene product optimal pH and temperature were pH 6-7 and 55–60°C, respectively, whereas for DB104 were pH 6-7 and 55°C, respectively (Figures 3(c) and 3(d)).

AQ1 recombinant xylanase seemed relatively more stable than DB104 one, for instance, after 30 minutes of incubation at 50°C it still retained its 52% activity compared to 29% residual activity of DB104 recombinant endoxylanase (Figure 3(e)).

The apparent molecular mass of AQ1 recombinant endoxylanase is considered to be 21 kDa, as showed in SDS-PAGE electrophoresis the band with this size was intensified with respect to the arabinose concentration used in the culture. This molecular mass of the endoxylanase agreed with the theoretically predicted value calculated for the deduced polypeptide (Figure 3(b)).

3.6. The Distribution of Recombinant Enzymes in Cellular Fractions in E. coli. As outlined in Materials and Methods, cytoplasmic, periplasmic, and extracellular fractions were subjected to the endoxylanase assay, and all of the fractions showed endoxylanase activity. The percentage of total endoxylanase activity of the cytoplasmic fraction in LB grown cells (without xylan) after 16-hour cultivation was rather high, more than 45% (Figure 4). However, when xylan was added to the medium, the extracellular fraction was found to contain the highest amount, and the cytoplasmic and periplasmic fraction tended to decrease. It seemed that the increase of total endoxylanase activity of supernatant depends on the xylan concentration. For example, after 24 hours of cultivation in LB without xylan, the amount of total activity of supernatant was 14,671 U of whereas the total activity increased to 19,392 and 20,583 U in the presence of 0.1% xylan and 1% xylan, respectively (Figure 4). There was a trend that the increase of total amount of enzyme activity in supernatant culture (out of the cells) was followed by the decrease in cytoplasmic and periplasmic fraction.

4. Discussion

We have successfully isolated a potent xylanolitic bacterium from an Indonesian local habitat. Previously, we have carried out cloning and heterologous expression of an endoxylanase gene from a local *Bacillus licheniformis* using an *E. coli* plasmid promoter [8].

In this paper, we report on the cloning and heterologous expression of a family 11 xylanase gene (*xyn11*) including its indigenous promoter. The gene originating from a new *B. subtilis* isolate was characterized in detail.

In species determination, 16S rRNA gene sequence comparisons should be performed only in conjunction with phenotypic and phylogenetic properties because distinct species may exist with identical 16S rRNA gene sequences due to the high diversity of members of the *B. subtilis* species [18–20].

Though the endoxylanase-encoding gene and its putative promoter display 91% identity on the nucleic acids level or 95% identity on amino acid level, physiological and phylogenetic analyses assured the species determination; at

Table 2: Comparison of the activity at 55°C pH 7 of the supernatant from the fermentation broth after 24 hours of cultivation at 37°C in the different medium content of *B. subtilis* AQ1, *B. subtilis* DB104, and recombinant *E. colis* at the same OD OR cell number per ml. The given values in activity assays are the means of triplicates independent experiment (± the standard deviation).

No.	Medium	LB	LB xylan (1%)
(1)	Endoxylanase activity of supernatant from original *B. subtilis* strain AQ1	72.6 ± 1 U/mL (306.2 ± 4.2 U/mg)	141.9 ± 1 U/mL (529.7 ± 5.6 U/mg)
(2)	Endoxylanase activity of supernatant from original *B. subtilis* strain DB104	16.47 ± 0.1 U/mL (105.71 ± 0.9 U/mg)	35.7 ± 0.26 U/mL (286.0 ± 2.1 U/mg)
(3)	Endoxylanase activity of supernatant from rec *E. coli* (PGEMxynAQ1+promoter)	586.9 ± 13.9 U/mL (2173.6 ± 51.4 U/mg)	823.6 ± 3.5 U/mL (2745.3 ± 11 U/mg)
(4)	Endoxylanase activity of supernatant from *E. coli* (PGEMxynDB104+promoter)	126.2 ± 3.9 U/ mL (573.6 ± 17.7 U/mg)	184.9 ± 5.2 U/mL (660.3 ± 18.6 U/mg)
(5)	Endoxylanase activity of supernatant from *E. coli* (PGEMxynAQ1-promoter)	N.D.	4.6 ± 0.4 U/mL (20.0 ± 1.7 U/mg)
(6)	Endoxylanase activity of supernatant from rec *E. coli* (PGEM T easy only)	N.D.	N.D.

*N.D.: no activity was detected at standard assay condition.

the same time such divergences clearly delineate *B. subtilis* AQ1 from the completely sequenced *B. subtilis* 168 and other totally 14 sequenced representatives belonging to the same species (not shown).

The 101 bp preceding the structural endoxylanase gene, presumably containing the indigenous respective promoter of *B.subtilis* AQ1, is evidently capable of driving transcription in *E. coli* since it facilitated the observed very high level of constitutive expression. This promoter region was 9 bp shorter than strain DB104 endoxylanase promoter region. This difference might cause different activity of the endoxylanase produced, since DB104 endoxylanase has lower activity. The detection of such significant levels of endoxylanase activities in the supernatant revealed that the cloned gene and its promoter, and presumably the signal peptide were recognized and it functioned in *E. coli*, so that it can facilitate the secretion into supernatant.

There was activity of *E. coli* DH5 α with pGEM-T easy with ORF of AQ1*xyn* minus promoter region which was grown in LB xylan, but it was insignificant compared to the recombinant with the promoter region. Thus, it is becoming evident that the high level expression of this AQ1 endoxylanase gene in *E. coli* was caused by the presence of native promoter, although from prediction the probability of the native promoter was only 50%.

The properties of the AQ1 and DB104 such as optimal pH and temperature are nearly the same; however the activity—both volumetric and specific activity—of AQ1 recombinant xylanase was higher. The thermostability of AQ1 recombinant xylanase was also relatively higher at 50, 55, and 60°C. The result suggested that 6 amino acid differences between AQ1 and DB104 xylanase in mature protein might contribute to this difference in thermostability. At the previous study, using direct evolution method it needed only 3 mutations in mature protein to improve the thermal stability of endoxylanase [21]. In fact, the concentration of AQ1 recombinant xylanase was also higher than that of DB104 that might also contribute to its higher thermal stability.

There was another paper reporting the cloning of xylanase gene from *B. subtilis* [12]. Different to our result, this recombinant endoxylanase gene was expressed in *E. coli* with the highest endoxylanase activities being located in the periplasmic fraction [12]. We obtained the highest endoxylanase levels in the supernatant, especially in the presence of xylan, possibly due to extended lyses of cells which may suffer from massive secretion of the enzyme to the periplasmic space eventually resulting in disintegration of the cell envelope.

The secretion into culture medium although without xylan was also reported by Ruller et al. 2006 [22]. Compared to the above study where they gained 216,000 U from 1 litre LB after 48 hours at 37°C, we obtained 20,583 U of recombinant endoxylanase in culture supernatant from 25 mL medium in less than 48 hours. It is noteworthy to state, however, that we—other than Ruller et al. 2006—supplemented the LB medium with xylan in laboratory scale.

The example secretion of hydrolytic enzymes mannanase, chitinase, and alpha amylase originated from *Bacillus* sp. into culture medium using native signal peptide but utilizing T7 promoter in *E. coli* as a host was also reported by Yamabhai et al. 2008 [23].

The total activity of the supernatant fraction of the recombinant endoxylanase producer increased when xylan was added; however, the activities in the cytoplasmic and in the periplasmic fraction decreased. As the induction of *B.subtilis* degradative enzymes is subject to gene regulation [24] which differs significantly from that in the Gram negative cloning host, the molecular basis or the reasons of such phenomenon is not yet understood and needs to be elucidated. A possible explanation might come from the secretion stress along with the concomitantly occurring osmotic pressure in xylan-containing media acting on the Gram negative host cells which eventually may lead to the breakdown of the cell wall and the outer membrane, finally releasing the enzyme to the environment (supernatant). Anyway, irrespective of the mechanisms the addition of substrate xylan significantly increased the yield and thus

can be applied for obtaining high endoxylanase activity in the culture supernatant. It needs to be elucidated, however, whether cost-effective and naturally available xylan-rich media can similarly be used for production purposes.

Apart from the specific application for xylanase production, the promoter region and signal peptide of this particular gene might be also applied for constitutive extracellular expression of other enzyme genes to such high level.

5. Conclusion

The endoxylanase gene including its indigenous promoter from newly isolated *B.subtilis* AQ1 strain has been cloned and expressed, and the gene product was secreted into supernatant of culture medium at high level. The AQ1 endoxylanase native promoter was 9 bp shorter than that of *B. subtilis* DB104 and its ORF had 95% homology at amino acid level with the last mentioned. The specific and volumetric activity of this recombinant enzymes was very high exceeding that of the recombinant DB104 endoxylanase. The presence of xylan increased the amount of enzymes secreted to the medium. This research showed the feasibility of *E. coli* as a host to produce heterologous extracellular enzymes gene using this AQ1 xylanase promoter and signal peptide.

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