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Cloning of α-L-arabinofuranosidase Genes and Its Expression in *Escherichia coli*: A Comparative Study of Recombinant Arabinofuranosidase Originating in *Bacillus subtilis* DB104 and Newly Isolated *Bacillus licheniformis* CW1

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Arabinofuranosidase (Abfa) is one of the most important enzymes involved in degradation of lignocelullose biomass. Two genes encoding α-L-Arabinofuranosidase (*abfA*), each from *Bacillus subtilis* DB104 (*abfAa1*) and an indigenous Indonesian *B. licheniformis* CW1 (*abfAb3*), were cloned by the PCR approach and expressed in *Escherichia coli*. Sequences analysis of *abfAa1* and *abfAb3* revealed that each consists of 1721 and 1739 base pairs long DNA, respectively. Each clone contains a hypothetical open reading frame of 1503 and 1509 bp that encode an Abfa protein of 500 and 502 amino acids for *abfAa1* and *abfAb3*, respectively. The deduced amino acid sequence of AbfaB3 shares 75% identity to that of AbfaA1. The recombinant enzymes were expressed constitutively in *E. coli*. Partial characterization of those enzymes revealed that the AbfaA1 and AbfaB3 were optimally active at 50 ºC and 60 ºC at pH 6, respectively. Thermostability studies of the recombinant enzymes with p-nitrophenyl α-L-arabinofuranoside at their optimal conditions showed that up to 50% AbfaA1 activity was lost after 5 h incubation at 50 ºC, whereas the AbfaB3 retained its activity over 75% after 12 h pre-incubation at 60 °C. This thermostability study of recombinant AbfaB3 showed for the first time that the arabinofuranosidase from *B. licheniformis* is a thermostable enzyme. The recombinant enzyme showed a higher optimal reaction temperature (60 ºC) in comparison to the previously reported thermostable arabinofuranosidase. The thermostable AbfaB3 has a potential to be applied to the degradation of lignocellulose biomass synergistically with thermostable xylanases, for instance in the production of xylo-oligosaccharides.

Key words : α-L-arabinofuranosidase, *Bacillus,* cloning, thermostability

Arabinofuranosidase merupakan salah satu enzim penting dalam degradasi biomassa lignoselulosa. Gen yang mengkodekan α-L-Arabinofuranosidase (*abfA*) masing - masing dari *Bacillus subtilis* DB104 (*abfaA1*) dan isolat asli Indonesia *B. licheniformis* CW1 (*abfaB3*) telah diklon dengan pendekatan PCR dan diekspresikan dalam *Escherichia coli*. Analisa sekuen *abfAa1* dan *abfAb3* menunjukan bahwa masing-masing klon secara berurutan mengandung DNA berukuran 1721 dan 1739 bp. Masing-masing klon DNA terdiri dari satu hipotetis kerangka pembacaan terbuka berukuran 1503 dan 1509 bp yang mengkodekan protein Abfa berukuran 500 dan 502 asam amino secara berurutan untuk klon AbfAa1 dan AbfAb3. Urutan asam amino hasil deduksi AbfaB3 mempunyai kemiripan 75% terhadap AbfaA1. Enzim rekombinan diekspresikan secara konstitutif pada *E. coli*. Karakterisasi parsial dari enzim tersebut menunjukkan bahwa AbfaA1 dan AbfaB3 masing-masing bekerja secara optimal pada suhu 50 ºC dan 60 ºC pada pH 6. Studi termostabilitas enzim rekombinan menggunakan *para*-nitrophenyl α-L-arabinofranoside pada kondisi optimalnya memperlihatkan bahwa aktivitas AbfaA1 turun hingga 50% setelah diinkubasi selama 4 jam pada suhu 50 ºC, sementara AbfaB3 mempertahankan aktivitasnya hingga lebih dari 75% setelah 12 jam inkubasi pada suhu 60 ºC. Studi termostabilitas AbfaB3 rekombinan ini memperlihatkan untuk pertama kalinya bahwa arabinofuranosidase dari *B. licheniformis* merupakan enzim termostabil. Enzim rekombinan ini memperlihatkan suhu optimal reaksi yang lebih tinggi dibandingkan dengan enzim termostabil yang telah dilaporkan sebelumnya. AbfaB3 termostabil ini potensial untuk digunakan dalam proses degradasi biomassa lignoselulosa secara sinergis dengan enzim xilanase termostabil dalam proses produksi xilooligosakarida.

Kata kunci: α-L-arabinofranosidase, *Bacillus*, kloning, termostabilitas

In the last few decades, bioconversion of lignocellulosic biomass has received a great deal of attention because of its potential application in various agro-industrial processes especially in the production of renewable biofuel (Keshwani and Cheng 2009; Sanchez and Cardona 2008) and chemicals such as xylo-oligosaccharides (Akpinar *et al*. 2009), protein (Bals *et al*. 2007), 2,3-butanediol (Saha and Bothast 1999), and ferulic acid (Hunhammar *et al*. 1997). Alpha-L-arabinofuranosidases (abfa, EC 3.2.1.55) are exo-type enzymes that catalyze the hydrolysis of the

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non reducing terminal α-L-arabinofuranosidic linkage in hemicellulose such as arabinoxylan, arabinan and other L-arabinose containing polysaccharide. These enzymes act synergistically with other hemicellulases, such as mannanase, xylanase, and acetyl xylan esterase to degrade the hemicellulose backbone completely (Ross *et al*. 1992; Gilead and Shoham 1995). As a debranching enzyme α-L-arabinofuranosidase is one of hemicellulases that seems to be critical in the early steps of hemicellulose degradation (Jeffries 1990).

Various attempts have been developed to obtain arabinofuranosidase enzymes including direct purification of those native enzymes (Kaneko *et al.* 1994; Gilead and Shoham 1995; Degrassi *et al*. 2003; Raweesri *et al*. 2008) as well as over-expressing their recombinant enzymes (Whitehead and Hespell 1995; Morales *et al*. 1995; Pei and Shao 2008). This view is supported by the genetic information accessible from GenBank (www.ncbi.nlm.nih.gov/genbank). Gene cloning and its expression in host cells such as *E. coli*, *Bacillus*, or yeasts have become a promising method of choice.

The objectives of the current study were to clone the two genes encoding α-L-arabinofuranosidase from *Bacillus subtilis* DB104 and a newly isolated and identified strain *B. licheniformis* CW1, as well as to express the cloned genes in *E. coli*. Characteristics of both recombinant enzymes were compared to select for a thermostable arabinofuranosidase having an optimum reaction temperature not less than 60 ºC, which has the potential to be applied synergistically along with thermostable xylanases in xylooligosacharide production from lignocellulosic biomass. This is usually conducted in a reaction temperature not less than 60 ºC.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions. *B. subtilis* DB104 and *B. licheniformis* CW1 were obtained from BPPT Culture Collection. The strains were grown at 37 °C, shaken at 150 rpm for 16-18 h in LB medium (pH 7.2) containing 10 g soy peptone, 10 g NaCl, and 5 g yeast extract per liter. For gene cloning and protein expression *E. coli* strain DH5α was employed and grown in LB medium containing ampicillin (100 μg mL^{-1}).

16S rDNA-Based Identification. *Bacillus* CW1 isolate was identified by 16S rDNA-based amplification using universal primers for eubacterial rRNA genes, 9F (5'-AGAGTTTGATC(C/A)TGGCTCAG-3') and 1510R (5'GTTAC(G/C)TTGTTACGACTT-3') (Dhiaf *et al*. 2008). The partial 16S rRNA gene sequence was analyzed and compared with known bacterial sequences in the NCBI GenBank using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and submitted to GenBank (www.ncbi.nlm.nih.gov/genbank).

Cloning of α-L-arabinofuranosidase-Encoding Genes. Genomic DNA was each extracted from *B. subtilis* DB104 and *B. licheniformis* CW1 according to the phenol/chloroform protocol developed by Sambrook and Russel (2001). The genes encoding α-L-arabinofuranosidases were each amplified by PCR from the genomic DNA using the following primer pairs: Abfabs-F (5'GACTAGTTAGTTCGGTCGAAAGAAATGTTTACGC-3') and Abfabs-R (5'-GAAGATCTTTA TGACTGTTTTTTCAGGCGGATCAC3') for *abfAa1*, Abfabl-F (5'ATGGTACCTAACGCTCCCAATCGGACTGT3') and Abfabl-R (5'GCGAGATCTTCATTGTTTCTTCATTCTGAT3') for *abfAb3*. Primers used were based on nucleotides sequences of α-L-arabinofuranosidase genes of *B. subtilis* 168 (GenBank NC_000964) and *B. licheniformis* DSM13 (GenBank NC_006322). The amplified genes were each cloned into pGEM-T Easy vector (Promega, Madison) according to the manufacturer's protocol. Positive clones were verified by restriction endonuclease analysis of the recombinant plasmids by using *Eco*RI and assays of their expressed recombinant protein against specific substrat para-nitrophenyl arabinofuranoside.

Sequence Analysis. Identification of the 16S rDNA and arabinofuranosidase genes were performed by using Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information) available at http://blast.ncbi.nlm.nih.gov/Blast.cgi with the standard parameters. Nucleotide sequences were aligned using Clustal W version 11 (Thompson et al. 1994) that is accessable as public web server at http://align.genome.jp/. The alignments were visually edited when necessary using GeneDoc (Nicholas and Nicholas 1997). The nucleotide sequence of the clone B3 containing the α-L-arabinofuranosidase gene of *B. licheniformis* CW1 was deposited in the GenBank database under accession number JN967641.

Enzyme Production. *E. coli* transformants harbouring recombinant plasmids containing arabinofuranosidase gene were cultivated in 50 ml LB media containing ampicillin. Cells were harvested from the overnight culture by centrifugation at 4025 x g (Rotor R10A, Hitachi) 4 ºC for 5 min. The pellet

obtained was resuspended in 5 mL citrate buffer pH 6, and disrupted by sonication (Heat system XL ultrasonicator) at the maximum frequency (20 kHz) for 20 s on and 20 s off repeatedly for 5 minutes at 4 °C. The crude extracts containing recombinant enzyme were then recovered by centrifugation for 10 min at 5800 x g.

Enzyme Assay. The arabinofuranosidase assay was based on the hydrolysis of *para*-nitrophenyl α-L-arabinofuranoside (pNP-A) (Sigma-Aldrich, USA) described by Martinez et al. (2006) with slight modification. The routine assay contained 175 µL of appropriately diluted enzyme sample, 175 µL of 50 mM citric acid-Na_2HPO_4 buffer (pH 6) containing 1 mM of pNP-A. Mixtures were incubated at 40 °C for 20 min, and the reaction terminated by the addition of 700 µl of 1 M Na_2CO_3. The colour intensity of the released *para*-nitrophenol was measured at 405 nm. One unit of activity was defined as the amount of enzyme that produces 1 µmol of p-nitrophenol per min under the assay conditions used (Martinez et al. 2006). The unit activity of enzyme is expressed in units of activity per mg of total protein (specific activity). The protein quantification was measured by the Bradford method with Bovine Serum Albumin (BSA) as standard (Bradford, 1976).

Effects of pH and Temperature on Arabinofuranosidase Activity. The effect of temperature on the activity of α-L-arabinofuranosidase was investigated in the reaction mixtures containing 50 mM citric acid-Na_2HPO_4 buffer (pH 6) by measuring the activity using pNP-A at specific temperatures ranging from 30-70 ºC. The activity of α-L-arabinofuranosidase over a pH range of 5-9 was analyzed by reacting the enzyme at their optimal temperatures in three different buffers (50 mM): citric acid-Na_2HPO_4 (pH 5 to 6), phosphate buffer (pH 7 to 8), Tris-HCl buffer (pH 9) (Gilead and Shoham 1995) using the routine assay. The results were expressed as a percentage of the activity obtained at either the optimum pH or the optimum temperature (Canakci 2007).

Thermostability. Temperature stability of α-L-arabinofuranosidase enzymes was studied by incubating them at their optimal temperatures in the absence of the pNP-A substrate. At various time intervals, an aliquot of the enzyme was removed and placed on ice. The residual enzymatic activity of each enzyme aliquot was determined by routine assay. The results were expressed as a percentage of residual activity calculated on the basis of the unheated sample. Enzyme thermostability was evaluated by determination of decimal reduction time (D-Value) and half life ($t_{1/2}$). D-value is defined as the time exposure required to reduce 90% of initial residual activity of arabinofuranosidase at a constant temperature (Wahyuntari and Suhartono 2002). The D-value was determined from the negative reciprocal of the slopes of the regression lines, using the linear portions of the residual activity versus time of exposure at constant temperature (as per Equation 1):

$$\mathrm{Log}\,(A) = \mathrm{Log}\,(A_0) - t/D \quad (1)$$

in which, A is the actual enzyme activity, A_0 is the enzyme activity before exposure to heat, t is length of time of heating, and D is decimal reduction time. The time at a specified temperature required for loss of 50% activity (half life) was calculated based on the equation 1 (Wahyuntari and Suhartono 2002).

RESULTS

16S rDNA-Based Identification. An indigenous bacterium CW1 isolate collected by BPPT-Cullture Collection was used as the genetic source in this work. This bacterial strain was isolated from Ciseeng Hot Spring, West Java, Indonesia. Partial sequencing of the amplified 16S rDNA of *Bacillus* CW1 isolate revealed 100% similarity to the 16S rDNA of *Bacillus licheniformis*. This molecular identification confirms the biochemical identification of the CW1 isolate, as previously identified as *B. licheniformis* (unpublished data). The partial sequence of the 16S rDNA has been submitted to the GenBank with accession number JN967640.

Cloning and Expression of α-L-Arabinofuranosidase Gene. In this work we have isolated α-L-arabinofuranosidase genes from genomic DNA of two *Bacillus* species by means of the PCR-cloning approach. Ampilification of arabinofuranosidase genes of *B. subtilis* DB104 (*abfAa1*) and *B. licheniformis* CW1 (*abfAb3*) revealed 1721 and 1739 bp DNA fragments, respectively. Restriction endonuclease analysis of the recombinant plasmids resulted in three DNA fragments consisting of undigested recombinant plasmids of 4736 or 4740 bp, a vector fragment of 2997 bp and the inserted fragment of 1739 or 1743 bp confirming succesfull gene cloning (Fig 1A). The cloned genes were each expressed and assayed to confirm their Abfa activity. The recombinant enzymes demonstrated positive activity towards the specific substrate *para*-nitrophenyl α-L-arabinofuranoside indicated by the

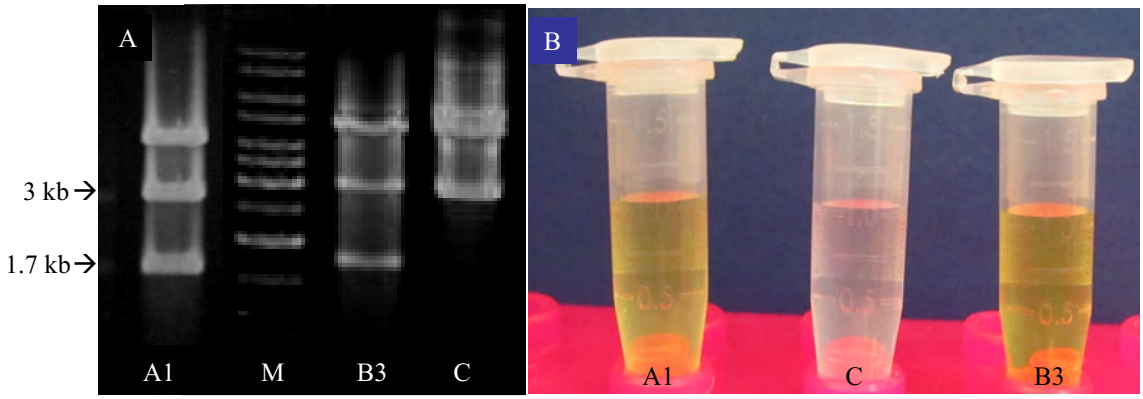

Fig 1 Verification of recombinant pGEM T easy plasmid harbouring *abfa* of *B. licheniformis* CW1 (A; line B3) and *B. subtilis* DB104 (A; line A1) by means of *Eco*RI restriction endonuclase analysis (A) and qualitative verification of the recombinant Abfa activity by means of assays against specific substrate *para*-nitrophenyl arabinofuranoside (B). M: 1 kb DNA marker; C: negative control.

color change of the reaction mixtures from clear to a slightly yellow (Fig 1B). Two positive clones (A1 and B3) were chosen for further analysis of their nucleotide sequences and biochemical properties of the expressed Abfas.

Sequence Analysis of *abfAa1* and *abfAb3*. Sequence analysis of the cloned *abfAa1* and *abfAb3* revealed the presence of an open reading frame of 1503 bp (*abfAa1*) and 1509 bp (*abfAb3*) for genes encoding a hypothetical protein of 500 and 502 amino acids of AbfaA1 and AbfaB3, respectively. Identification of those proteins by database enquiry using BLASTP tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi) determined that the 500 and 502 amino acids proteins were α-L-arabinofuranosidases which showed 100% identity to putative arabinofuranosidases of *B. subtilis* 168 and *B. licheniformis* ATCC 14580, respectively. The deduced amino acid sequence of AbfaB3 shares 75% identity with that of AbfaA1 (Fig 3). No putative signal sequence was detected when amino acid sequences of the AbfaA1 as well as AbfaB3 were analysed using signal prediction tools SignalP V1.1 (available as public web server at http://www.cbs.dtu.dk/service/SignalP/), indicating that both arabinofuranosidases are intracellular enzymes.

Partial Characterization of Recombinant Arabinofuraosidases. Effect of temperature and pH on activity and stability of the recombinant Abfa were studied. The AbfaA1 and AbfaB3 were active at pH range of 6-8 and temperature of 30-70 ºC. AbfaB3 was relatively more tolerant to pH in comparison to AbfaA. AbfaB3 retained its enzymatic activity in the pH range of 6-9, whereas that of AbfaA1 was active in the pH range of 6-8; however both enzymes showed maximum activity at pH 6 (Fig 2A). The enzymatic activity of AbfaA1 seemed to be relatively stable at temperatures below 50 ºC, but it decreased drastically at temperatures above 50 ºC (Fig 2B). In contrast, enzymatic activity of AbfaB3 increased along with the increased reaction temperature to reach its maximum activity at 60 ºC. When incubated at their optimal pHs and temperatures, AbfaA1 (50 ºC) retained less than 60% activity after 4 h (Fig 2C) while AbfaB3 (60 ºC) maintained more than 75% activity after 12 h. Employing the equation (1), the following inactivation equations of AbfaA1 and AbfaB3, respectively were obtained:

$$\text{Log}(A) = -0.0475(t) + 0.3385 \text{ for AbfaA1 at } 50\,^{\circ}\text{C} \quad (2)$$

$$\text{Log}(A) = -0.0095(t) + 0.6000 \text{ for AbfaB3 at } 60\,^{\circ}\text{C} \quad (3)$$

based on equations (2) and (3), the D-value of AbfaA1 at 50 ºC and AbfaB3 at 60 ºC are 8 and 63 h, respectively. The half life of both Abfas estimated by the corresponding equations (2) and (3) revealed $t_{1/2}$ values of 2 h (AbfaA1) and 19 h (AbfaB3). The predicted $t_{1/2}$ value of AbfaA1 seemed to be shorter than the actual experimental data by about 4 h (Fig 2C).

DISCUSSION

A few arabinofuranosidases have been purified and characterized from the genus *Bacillus*. Examples are *B. pumilus* (Degrassi *et al*. 2003), *B. polymyxa* (Morales *et al*. 1995), *B. stearothermophilus* (Gilead and Shoham 1995; Bezalel *et al*. 1993), and *B. subtilis* (Kaneko *et al*. 1994). To our knowledge, this is the first report on an arabinofuranosidase characterization isolated from *B. licheniformis*. In this work, an arabinofuranosidase gene, *abfaB3*, was successfully isolated and cloned from *B. licheniformis* CW1. Identification of the arabinofuranosidase gene worked well using gene

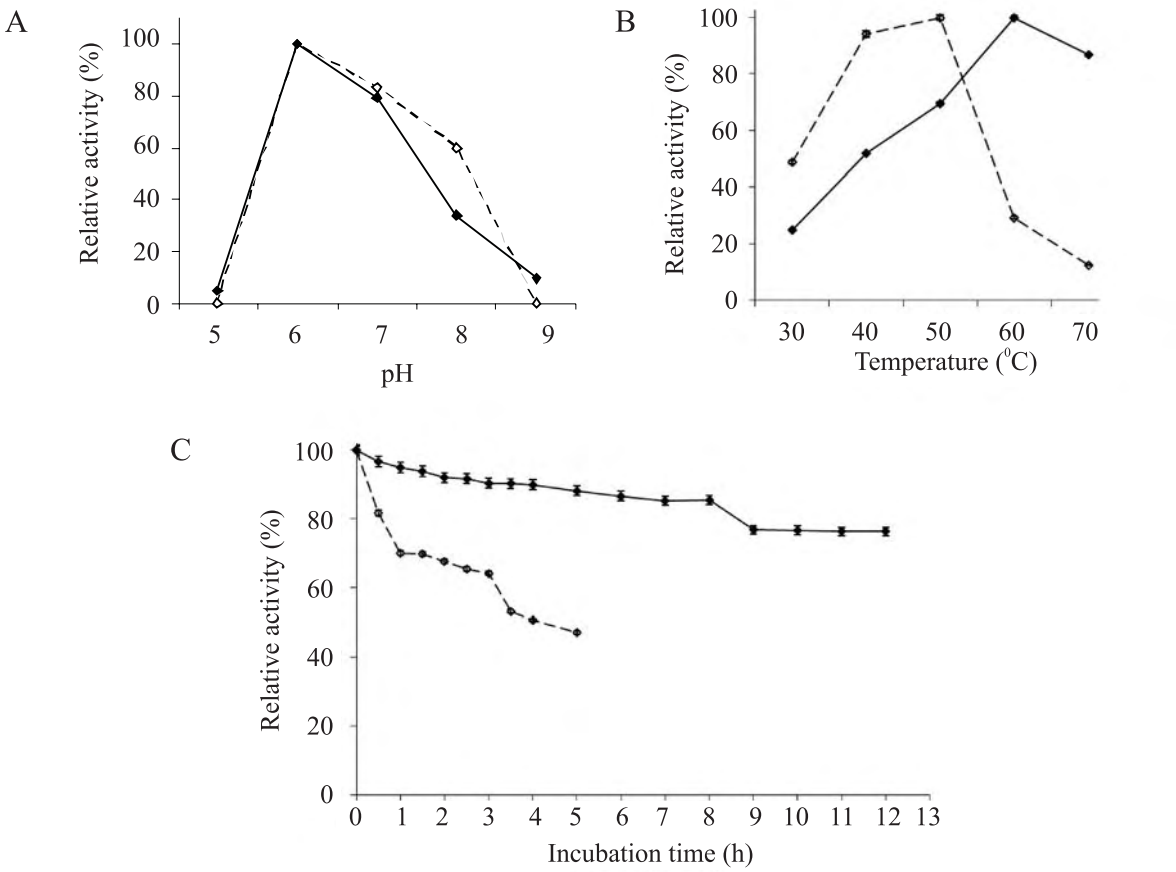

Fig 2 Properties of crude extracts of recombinant arabinofuranosidase originated from *B. subtilis* DB104, AbfaA1 (--◊--) and *B. licheniformis* CW1, AbfaB3 (—◆—). (A) The effect of pH on the recombinant Abfa activity in different buffers. The reaction pH range were adjusted between 5 – 9 with the following buffers: 50 mM citrate buffer (pH 5 to 6), phosphate buffer (pH 7 to 8) , Tris-HCl buffer (pH 9). The activity at optimal pH was defined as 100%. (B) The effect of temperature on the activity of recombinant Abfa. The activity at optimal temperature was defined as 100%. (C) The thermostability of recombinant Abfa at optimal pH and temperature. The initial activity was defined as 100%. Standard deviations are indicated by bars.

specific primer (GSP) designed based on a putative α-L-arabinofuranosidase of *B. licheniformis* ATCC 14580. A similar result was observed in the PCR cloning of arabinofuranosidase gene of *B. subtilis* DB104 using a pair of GSPs derived from a putative alpha-L-arabinofuranosidase gene of *B. subtilis* 168. This result indicates that the gene is highly conserved between strains of the same species, as previously demonstrated by Park *et al.* (2007), by means of degenerate primer PCR approach to amplify arabinofuranosidase gene from *Bacillus*-associated species.

The arabinofuranosidase activity was detected in the cytoplasmic fraction of recombinant *E. coli* containing either the *abfAa1* or *abfAb3* gene, which had been confirmed not to contain signal peptides, as predicted *in silico* by SignalP IV tools (http://www.cbs.dtu.dk/services/SignalP/). This was in accordance with the subcellular localization analyses of recombinant AbfA and abf2 activities reported by Inacio *et al.* (2008), which showed that both recombinant proteins were retained in the cytoplasm of *B. subtilis*. However, Abfa activity was detected in the cell-free culture broth of *B. subtilis* DB104 as well as *B. licheniformis* CW1 (data not shown) as was previously reported in *B. subtilis* 3-6 by Kaneko *et al.* (1994), in *B. stearothermophilus* T-6 by Gilead and Shoham (1995), and in *B. pumilus* by Degrassi *et al.* (2003). This phenomenon is in agreement with the study of *B. subtilis* extracellular proteome that indicates the presence of some extracellular enzymes without signal peptide (Antelmann *et al.* 2001). In 2005 Bendtsen *et al.* proposed a non-classical protein secretion pathway to elucidate the presence of some functional cytoplasmic proteins in growth medium of some bacteria cultures such as *M. tuberculosis* (Hart and Horwitz 1997), *Streptococcus pyrogenes* (Rosch and Caparon 2004), and *B. subtilis* (Hirose *et al.* 2000). These non-classical secreted proteins often seem to have a cytoplasmic function as well as an extracellular

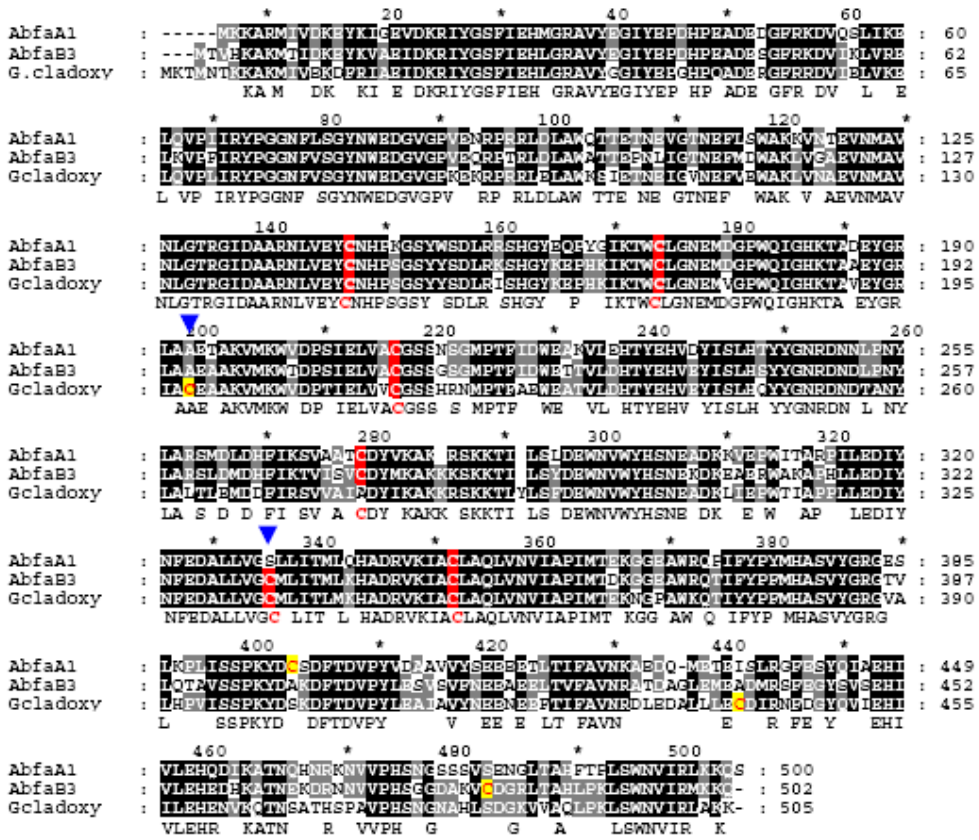

Fig 3 Alignment of the deduced amino acid sequences of the α-L-arabinofuranosidase of *B. subtilis* DB104 (AbfaA1) and *B. licheniformis* CW1 (AbfaB3). Identical amino acids are indicated by black shades. Hyphens indicate gaps. Consensus sequence indicated below is based on the allignment of AbfaB3, AbfaA1 and putative Abfas of *B. amyloliquefaciens* TA208 (NCBI-GI: 328554491), *B. halodurans* (NCBI-GI: 15614424), *B. atrophaeus* (NCBI-GI:311066968) as well as the known Abfas of *Geobacillus stearothermophilus* (NCBI-GI:122937809) and *G. cladoxylolyticus* (NCBI-GI:113374907). The blue arrow head (▼) indicates the Cys positions assumed to be crucial in the protein stabilization. The red blocked C (C) indicates conserved Cys whereas the yellow blocked Cys (C) indicates the unique Cys of the corresponding arabinofuranosidase.

role. However, in the case of *B. licheniformis* CW1 Abfa, cell lysis during exponential growth or during entry into the stationary phase cannot be excluded as a possible reason for the Abfa activity detected in the extracellular millieu. Further analyses such as subcellular localization or a control study using a cytoplasmic marker protein, such as isocitrate dehydrogenase (Gilead and Shoham 1995), should be conducted to discriminate the Abfa activity caused by cell lysis or secretion. Gonzales-Pastor *et al*. (2005) observed that *B. subtilis* is able to initiate lysis to surrounding cells as a mechanism to postpone sporulation. The activity of the two enzymes towards *para*-nitrophenyl-arabinofuranoside in various pH and temperature conditions shows that AbfaB3 is more stable at higher temperature (60 ºC) in comparison to AbfaA1 (50 ºC). AbfaB3 is optimally active at 60 ºC which is higher than the optimal temperature reaction of a thermostable Abfa from *B. pumilus* (55 ºC; Degrassi *et al*. 2003), *B. polymyxa* (55 ºC; Morales *et al*. 1995), or *Butyryvibrio fibrisolvens* (50 ºC; Hespell *et al*. 1992), but lower than that of *B. stearothermophilus* L1 and T-6 (70 ºC; Bezalel *et al.* 1993; Gilead and Shoham 1995), *Geobacillus caldoxylolyticus* TK4 (75-80 ºC; Canakci *et al*. 2007), *Thermotoga maritima* MSB8 (90 ºC; Miyazaki 2005), and *Thermobacillus xylanilyticus* (90 ºC; Debeche *et al*. 2000). Based on these data it can be concluded that in terms of temperature stability, AbfaB3 is the second best enzyme among *Bacillus* arabinofuranosidase with a D-value of 63 h and half-life of 19 h.

The primary structures of AbfaA1 and AbfaB3 share 71% and 75% identity respectively to that of the characterized thermostable Abfa of *G. caldoxylolyticus* TK4 (Canakci *et al*. 2007). When the amino acid sequence of AbfaA1 and AbfaB3 were compared to that of *G. cladoxylolyticus* TK4 (AbfaTK4) in terms of cysteine content, AbfaA1 has one less cysteines (6 Cys) than AbfaB3 and AbfaTK4 (7 Cys). All of them contain four conserved Cys residues (Cys147, Cys174,

Cys216, and Cys351). The remaining non conserved Cys residues are located in different positions within each Abfa. One cysteine residue (Cys198) has a location which indicates it is essential in stabilizing the Abfa of *G. cladoxylolyticus*. This Cys198 is altered to alanine (Ala198) which is conserved among seven different Abfa's including AbfaA1 and AbfaB3 (Fig 3). This alteration (A198C) may lead to a possible formation of disulfide bond between Cys198 and Cys216 which could stabilize the protein conformation of AbfaTK4 resulting in 10 or 20 ºC higher thermostability in comparison to AbfaB3 or AbfaA1, respectively. A similar phenomenon is observed between AbfaB3 and AbfaA1 sequences. One cysteine residue (Cys335) which is conserved in AbfaTK4 as well as AbfaB3, is altered to alanine (A335) in AbfaA1. This alteration (C335A) is most probably responsible for the lower thermostability of AbfaA1 in comparison to AbfaB3, as the result of a possible loss of the disulfide bond formed between Cys335 and cys351. However further studies on site directed mutagenesis of either *abfAa1* or *abfAb3* should be carried out to support this hypothese since cysteine content is not the only factor to contribute to protein thermostabilization. The important role of Cys in the protein thermostability has been reported in the case of α-amylase of *Pyrococcus furiosus* (Savchenko 2002), endoglucanase Cel12A of *Humicola grisea* (Sandgren *et al*. 2003), and manosidase of *Aspergilus satoi* (Tatara *et al*. 2005). In all these studies it is evident that substitution at Cys residue greatly decreased thermostability of the enzyme. In other studies, cysteine residues were introduced to form *de novo* disulfidebridges in order to improve thermostability of endoxylanase of *T. reesei* (Fenel *et al*. 2004) and *B. stearothermophilus* (Jeong 2007). Cysteine frequently forms a disulfide bond that stabilizes proteins by lowering their conformational entropy compared with their unfolded state (Zhang *et al*. 2011).

Based on its relatively higher thermostability, the newly isolated and characterized arabinofuranosidase of *B. licheniformis* CW1 is potential to be applied in the degradation process of lignocellulose biomass in combination with thermostable xylanases, which usually conducted at high temperature (not less than 60 ºC) to produce xylooligosaccharides.

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