ORIGINAL PAPER

A thermophilic *α*-L-Arabinofuranosidase from *Geobacillus vulcani* GS90: heterologous expression, biochemical characterization, and its synergistic action in fruit juice enrichment

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Abstract

$α$-L-Arabinofuranosidases with an orchestral action of xylanolytic enzymes degrades the xylan in plant cell wall. In this study, heterologous expression, biochemical characterization, and synergistic action of $α$-L-Arabinofuranosidase from previously identified.*Geobacillus vulcani* GS90 (*Gv*Abf) was investigated. The recombinant $α$-L-Arabinofuranosidase was overexpressed in *Escherichia coli* BL21 (λDE) and purified via His-tag Ni-affinity and size-exclusion chromatography. Optimum activity of the purified $α$-L-Arabinofuranosidase was obtained at pH 5 and at 70 °C. The *Gv*Abf was active in a broad pH and temperature ranges; pH 4–9 and 30–90 °C, respectively. In addition, it retained most of its activity after an hour incubation at 70 °C and remained relatively stable at pH 3–6. *Gv*Abf was quite stable against various metal ions. The kinetic parameters of *Gv*Abf was obtained as V_{max} and K_m; 200 U/mg and 0.2 mM with *p*-nitrophenyl-$α$-L-arabinofuranoside and 526 U/mg and 0.1 mM with sugar beet arabinan, respectively. The synergistic action of *Gv*Abf was studied with commercially available xylanase on juice enrichment of apples, grapes, oranges, and peaches. The best juice enrichment in terms of clarity, reducing sugar content, and yield, was achieved with *Gv*Abf and xylanase together compared to treatment with xylanase and *Gv*Abf alone in all fruits. The treatment with *Gv*Abf and xylanase together lead to an increased juice yield by 26.56% (apple), 30.88% (grape), 40.00% (orange) and 32.20% (peach) as well as having a significant effect on juice clarity by an increase of % transmittance 47.26, 25.98, 41.77, and 44.97, respectively. The highest reducing sugar level of fruit juices also obtained with *Gv*Abf and xylanase together compared to treatment with xylanase and *Gv*Abf alone in all types of fruits. *Gv*Abf and xylanase together as simultaneous synergistic manner may have an exciting potential for application in fruit juice processing.

Keywords $α$-L-Arabinofuranosidase · *Geobacillus vulcani* · Fruit juice clarification · Synergistic action

Introduction

Hemicellulose is one of the main components of plant cell wall and the second most abundant natural polysaccharide after cellulose [1]. To utilize hemicelluloses in a various biotechnological applications, the polymeric structure of hemicellulose has to be hydrolyzed into monosaccharides. Xylan, the main structure of hemicellulose, is degraded by an orchestral action of xylanolytic enzymes including $α$-L-Arabinofuranosidases (Abfs) (EC 3.2.1.55). The main role of $α$-L-Arabinofuranosidases is to break the terminal and non-reducing $α$-1,2-, $α$-1,3-, and/or $α$-1,5-L-arabinofuranoside substitutions into $α$-L-arabinosides such as arabinoxylan, L-arabinan and the other polysaccharides that include arabinose [2]. On the basis of amino acid sequence similarities and mode of action against different substrates, Abfs can be classified into five glycoside hydrolase (GH) families; GH3, 10, 43, 51, 54, and 62 [3].

Some reports have been documented about synergistic actions of Abf and other xylanolytic enzymes such as xylanase, β-xylosidase, acetyl esterase, and endo-(1,5)-$α$-L-arabinanase in a concerted manner against different type of heterogenous xylans. Accordingly, different combinations of enzyme coctail including Abf has a higher synergistic impact rather than individual Abf action [4–8]. Abf and xylanase together shows synergetically higher action than

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the combination of Abf and another hemicellulose against wheat arabinoxylan. This great synergy between α-L-Arabinofuranosidase and xylanase may be attributed that it can likely be one way for degradation of biomass using minimal enzyme loading [6].

Importantly, Abf and other hemicellulases like xylanase play synergistically a promising role in the agro-industrial process [5, 9, 10] containing beverage industry, wine industry, paper from pulp, increase of animal feedstuffs, improvement of bread and bioethanol production [11–15]. It has been shown that Abf and xylanase exhibit a synergistic manner in the pulp and paper industry using unbleached kraft pulp [16]. Another integration action among these enzymes has also shown in bioethanol production using wet arabinoxylan [6]. In this respect, although it is known that xylanase and Abf play a role individually on fruit juice clarification [17, 18] their synergistic action has not been reported for this process in beverage industry so far.

Abfs are widely distributed in bacteria, fungi and plants [4, 19, 20]. Recently, much effort has been devoted to identification of α-L-Arabinofuranosidases from extremophilic microorganisms, because the related industrial processes operate at high temperatures [21, 22]. For instance, in general, fermentation conditions are adjusted to minimum 60 °C, because high temperatures eliminate contamination problems and provide not only fluidic medium but also soluble substrates [23]. Up to date, many studies have been performed for isolation of Abfs from several sources [22, 24–28], but α-L-arabinofuranosidase activity has been shown in only three members of thermophilic *Geobacillus* genera; *Geobacillus stearothermophilus, Geobacillus thermoleovorans* and *Geobacillus caldoxylolyticus* [29–31]. So far, to our knowledge, *Geobacillus vulcani* (*G. vulcani*) has not been reported as a source for α-L-Arabinofuranosidase. Here, we reported the heterologous expression and biochemical characterization of α-L-Arabinofuranosidase from previously identified *G. vulcani* GS90 (*Gv*Abf) by 16S rRNA [32] for the first time. In addition, the synergetical action of *Gv*Abf with a commercially available xylanase was demonstrated as a novel application of apple, orange, grape and peach juice enrichment in the present study. Although *Gv*Abf was effective on fruit juice enrichment process including clarification, reducing sugar content and yield, utilization of *Gv*Abf with the xylanase enzyme as a synergistic manner displayed exciting potential for application in fruit juice processing.

Materials and methods

Materials

Sugar beet arabinan was purchased from Megazyme (Bray, Wirklow, Ireland). Genomic DNA Isolation Kit, PCR Amplification Kit, Gel Purification Kit, TA Cloning Kit and the restriction endonucleases were obtained from Fermentas (Fermentas-Life Science Technologies, Lithuania). All other chemicals including pNP-α-L-arabinofuranoside and endo β-1,4-xylanase were supplied by Sigma Chemical (St. Louis, MO, USA).

Bacterial strains and growth conditions

Previously identified *G. vulcani* GS90 (*Gv*Abf) by 16S rRNA [32] was used as a source of α-L-Arabinofuranosidase and *E. coli* DH5α was used for molecular cloning purpose. This strain was grown in Luria–Bertani (LB) medium in the presence of ampicillin (100 µg ml^{-1}). *E. coli* BL21 (λDE3) was used for expression of *Gv*Abf in the presence of kanamycin (30 µg ml^{-1}), chloramphenicol (20 µg ml^{-1}) and isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM) in LB medium. The bacterial strain used in this study was from Balçova Geothermal region of İzmir and the xylanolytic activity of the strain was screened previously [33].

Cloning of *Gv*Abf gene

*Gv*Abf gene was amplified from the genomic DNA of the bacterial strain using the following primers 5′-<u>catatg</u>ggc-tacaaaaaaagcaacc-3′ for forward and 5′-<u>aagctt</u>ttatcgttttc-ctaaacg-3′ for reverse with the *Nde*I and *Hind*III sites (underlined), respectively. The amplified *Abf* gene was first cloned into pTZ57R/T vector (Fermentas-Life Science Technologies, Lithuania) and transformed to *E. coli* DH5α. After plasmid isolation, the DNA fragment was digested with *Nde*I and *Hind*III enzymes, gel-purified and cloned into *Nde*I/*Hind*III restricted pET-28a(+) expression vector (Novagen). The nucleotide sequence of the gene in the pET-28a(+) plasmid was determined and deposited in the EMBL Nucleotide Sequence Databases with the assigned accession number of HE653772.

Expression and purification of the *Gv*Abf enzyme

Escherichia coli BL21 (DE3) harboring the IPTG inducible plasmid pET-28a(+) encoding for *Gv*Abf enzyme was co-expressed with a chaperon protein; pG-Tf2 (Chaperone Plasmid, TAKARA Bio Inc., Japan), to increase recovery of the correctly folded target enzyme. For this purpose, this strain was grown at 200 rpm, 37 °C in LB medium (100 ml) with convenient antibiotics. When the OD$_{600}$ reached to 0.5, co-expression is induced by addition of 1 mM IPTG and culture was more incubated at 225 rpm, 37 °C for 4 h. The cells were harvested by centrifugation at 10,000×g, 4 °C for 20 min. After resuspension of the cell pellet in 50 mM Na-P buffer pH 7.0, cell membranes were disrupted by sonication for 5 min on ice (Bandelin, Sonopuls Ultrasonic Homogenizers,

HD 2070). Then, the cell debris was discarded by centrifugation at 16,000*g*, 4 °C for 15 min. The supernatant was subjected to nickel affinity chromatography for protein purification. The nickel column (HIS-Select® Nickel Affinity Gel, Sigma) was washed with 10 column volumes of ice cold 20 mM Tris–HCl buffer, pH 8.0 (300 mM NaCl, 10 mM of imidazole), and the bound proteins were eluted with elution buffer (250 mM imidazole, 100 mM NaCl in Tris–HCl buffer, pH 8.0). The proper fractions were pooled and concentrated using a cut-off filter (Amicon® Ultra-4 Centrifugal Filter Unit), and further purified by size-exclusion chromatography using Sepharose G-100 resin. The purified sample was checked for the purity of the target protein by SDS–PAGE analysis (10%). Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method [34]. Bovine serum albumin was used as a standard protein.

Biochemical characterization of *Gv*Abf

Activity assay

Activity of *Gv*Abf enzyme was determined via the previously described method by Canakci et al. with some modifications [31]. Briefly, the enzyme was assayed using the mixture contained 25 µl of 2 mM *p*NP-α-L-arabinofuranoside (*p*NP), 425 µl of McIlvaine buffer (pH 6.0) and 50 µl of enzyme solutions (0.1 µg). The hydrolysis reaction was carried out at 50 °C for 10 min and then terminated by the addition of 500 µl of 1.0 M Na_2CO_3 solution. The released *p*NP amount was determined at 420 nm with the extinction coefficient value of 1.78×10^4 M^{-1} cm^{-1}. One unit of enzyme activity is defined as the amount of enzyme that releases 1 µmol of *p*NP per minute.

Sugar beet arabinan (2%, w/v) was also used as substrate to investigate the ability of *Gv*Abf to release arabinose. For this purpose, the same assay procedure was used as described for *p*NP-α-L-arabinofuranoside, exceptionally at pH 5.5 and 65 °C. The dinitrosalicylic acid (DNS) method [35] was performed to investigate the reducing sugar content at the end of the reaction where L-arabinose was used as the standard. One unit of enzyme activity is defined as the amount of 1 µmol of arabinose that released by the enzyme per minute.

Optimum pH and temperature for enzyme activity

The effect of pH on enzyme activity was investigated using buffers at different pH, ranging from 3 to 12; Na–citrate buffer (pH 3–6), sodium phosphate buffer (pH 7–8), and glycine–NaOH buffer (pH 9–12). The optimum pH of the enzyme was determined under the standard assay conditions.

The effect of temperature on enzyme activity was examined under standard assay conditions at different temperatures (30–90 °C) using the optimum buffer and the buffer was heated to the relevant temperature before the assay.

pH and temperature stability

*Gv*Abf enzyme was also characterized by means of pH and temperature stability. To investigate the temperature stability, the enzyme was incubated in Na–citrate buffer (pH 5) at 50, 60, 70, 71, 72 and 75 °C for 1 h and the residual enzyme activity was measured after each incubation using standard activity assay. For the pH stability, *Gv*Abf enzyme was incubated for 1 h at 70 °C in buffers; pH 3–10, and then the residual enzyme activity was determined by the standard activity assay.

Effect of metal ions and chemical reagents on the *Gv*Abf activity

To assess the influence of metal ions ($CuSO_4$, KCl, $CaCl_2$, $MgCl_2$, $MnCl_2$, $CdSO_4$, NaF, $CoCl_2$, NaCl and $NiCl_2$) and chemical reagents (DTT, EDTA and BME, ethanol, hexane, methanol, ethyl acetate, acetone, acetonitrile), *Gv*Abf enzyme was incubated in the presence these chemicals at room temperature for 1 h. The reaction was initiated by addition of *p*NP-α-L-arabinofuranoside and then the remaining enzyme activity was determined by the standard activity assay.

Kinetic studies

Kinetic parameters of *Gv*Abf enzyme were determined using Lineweaver–Burk plots and assuming that the reactions followed a simple Michaelis–Menten kinetics. Lineweaver–Burk plots were obtained both for *p*NP-α-L-arabinofuranoside and for sugar beet arabinan using standard activity assay.

Enzymatic hydrolysis in fruit juice enrichment

*Gv*Abf enzyme was tested for its ability to enrich fruit juices in terms of clarity, yield end reducing sugar content. In addition, the synergetical action of *Gv*Abf was investigated with a commercially available xylanase in fruit juice clarification, yield and reducing sugar content processes in a similar way. Fresh fruits (apple, orange, grape and peach) were used for preparing fruit juice. They were collected from local vendor, carefully washed, dried in air and homogenized. Homogenized fruit juices were treated with 1 mg/ml of pure *Gv*Abf enzyme at 50 °C for 4 h. At the end of treatments, the juice samples were centrifuged at 5000*g* for 10 min. Supernatants

were analyzed for clarity, reducing sugar and yield. The clarity of the fruit juices was determined by measuring the percent transmittance (%T) at a wavelength of 660 nm using UV–Vis spectrophotometer. The percent transmittance was considered a measure of juice clarity. The yield of juices was detected by measuring the volume of the fruit juice after centrifugation. Furthermore, release of reducing sugars from fruit juices was examined by DNS method [35, 36]. To compare the fruit juice enrichment effect of xylanase, the similar studies were performed with a commercially available endo β-1,4-xylanase (Sigma) under the same conditions. Each fruit juices after homogenization were treated with 20 IU/ml endo β-1,4-xylanase at 50 °C for 4 h. At the end of treatments, the juice samples were centrifuged and analysis of clarity, reducing sugar and yield were performed like GvAbf application. The synergistic effect of GvAbf enzyme with endo β-1,4-xylanase was also studied for the same fruit juice enrichment tests including clarity, yield and release of reducing sugar level. Both enzymes (1 mg/ml of pure GvAbf and 20 IU/ml endo β-1,4-xylanase) together were applied to the indicated fruit juices using the same procedures and analysis of clarity, reducing sugar and yield were determined.

Data presentation and statistical analysis

All experiments were conducted in triplicate. Statistical errors of the data were determined using Original Software, version 5.0 Original Lab and Excel Software, Microsoft.

Bioinformatic analysis

In this study, amino acid sequence alignment was used by BLAST to investigate the similarity of GvAbf with the other Abf enzymes from different sources. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 [37].

Results and discussion

Hemicelluloses are mainly composed of xylans, and degradation of this component is dependent on the orchestral action of xylanolytic enzymes such as Arabinofuranosidases and xylanases [2]. We reported an α-L-Arabinofuranosidase from previously identified thermophilic bacterium *G. vulcani* GS90 [32] which releases arabinose moiety from sugar beet arabinan. After the enzyme characterization, we investigated the synergetical action of GvAbf in fruit juice enrichment process.

Amino acid sequence of *Gv*Abf

GvAbf gene was isolated from chromosomal DNA of *G. vulcani* GS90 and its length was determined as 1509 bp encoding 502 amino acids. Based on the sequence analysis, it was shown that GvAbf is an α-L-Arabinofuranosidase belonging to GH51 family. Significantly, GvAbf has a 98.6% amino acid sequence identity to the α-L-Arabinofuranosidase from *G. stearothermophilus* T-6. GvAbf also has Glu175 (acid–base catalytic residue) and Glu294 (catalytic nucleophile residue) which were reported to be responsible for the catalytic activity [38]. In addition, GvAbf showed 89.7% identity to α-L-Arabinofuranosidase from *G. caldoxylosilyticus* [31]. Yet, there was low identity between GvAbf and α-L-Arabinofuranosidase from *T. xylanilyticus* by 26.3% [21]. The phylogenetic tree was constructed considering the amino acid sequences of the α-L-Arabinofuranosidases in GH51 family by maximum likelihood (ML) method to determine the evolutionary connection of GvAbf. Apparently, GvAbf had the closest connection with α-L-Arabinofuranosidase of *G. stearothermophilus* T-6 (Fig. 1).

Expression and purification of *Gv*Abf

GvAbf, consisting of 502 amino acids was co-expressed in the presence of a chaperon protein pG-Tf2 as described in the method section. The GvAbf was purified to homogeneity using conventional Ni–NTA affinity purification followed by size-exclusion chromatography. The recovery of

Fig. 1 The phylogenetic tree showing the relation of Gv Abf with the other α-L-Arabinofuranosidases from the GH51 family
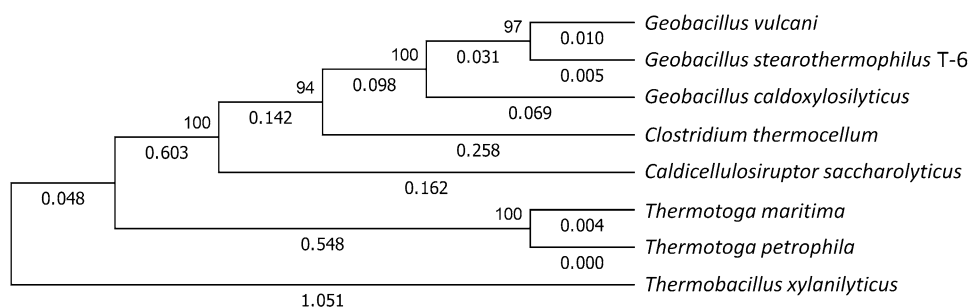
the expressed *Gv*Abf in the soluble fraction was increased significantly by the help of a chaperon protein. The sample purity was assessed by SDS–PAGE analysis confirming the molecular mass of the *Gv*Abf around 60 kDa (Fig. 2).
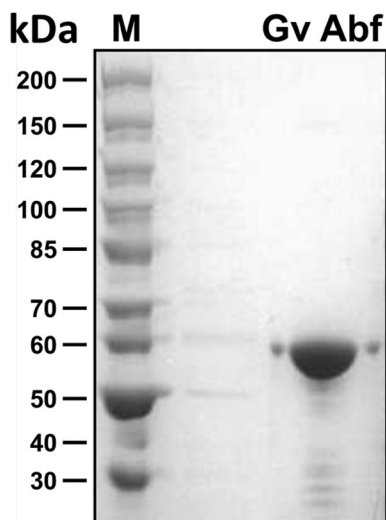

Fig. 2 The SDS–PAGE gel image of the purified Gv Abf

Biochemical characterization of *Gv*Abf

Purified recombinant *Gv*Abf was characterized and the results were summarized in Fig. 3 and Tables 1 and 2. The *Gv*Abf activity were evaluated in the temperature interval of 30–90 °C and pH interval of 3–12 (Fig. 3a, b). The enzyme showed hydrolytic activity in broad pH and temperature range, and the maximum activity was observed at 70 °C and pH 5. In addition, the effect of different pH and temperature values on the *Gv*Abf stability were determined (Fig. 3c, d). *Gv*Abf was quite stable at at high temperatures and the thermal stability tests showed that the enzyme remained relatively stable even after 1 h of incubation at 70 °C without a major activity loss (~90%). In terms of pH stability, *Gv*Abf had an interesting profile. The enzyme was stable at pH 4, 5, 8 and 9 after 1 h of incubation at 70 °C; however, at pH 6 and 7, after 30 min incubation, it started to lose its activity. Especially, the enzyme activity was reduced by half at pH 6, and almost 80% was lost at pH 7 after 1 h of incubation (Fig. 3d). In the case of pH stability, *Gv*Abf exhibited better profile under weak acidic conditions than neutral and alkaline pH values. At optimum pH and temperature values, *Gv*Abf retained 90% of activity after 1 h of incubation. α-L-Arabinofuranosidase from *G. caldoxylolyticus* TK4 kept its
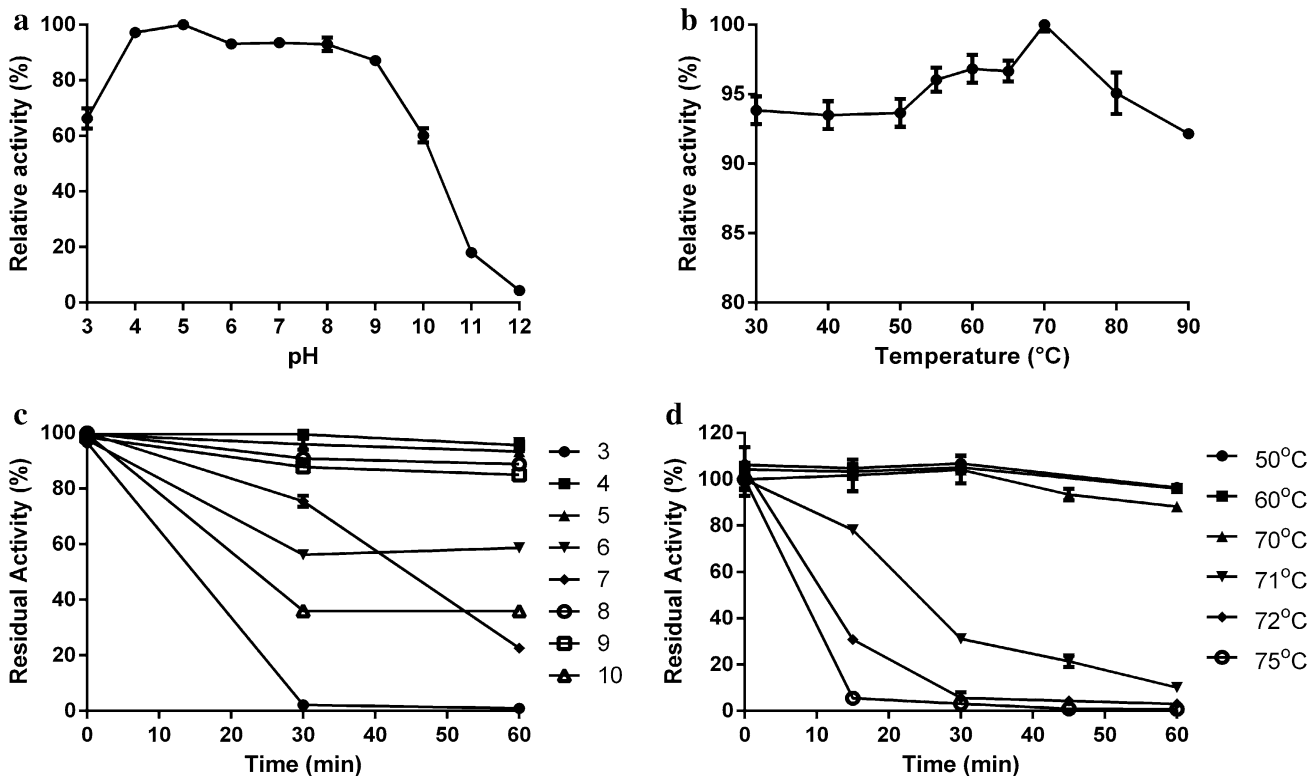

Fig. 3 Effects of pH and temperature on the activity and stability of the purified Gv α-L-Arabinofuranosidase. **a** Effect of pH, **b** temperature on the *Gv*Abf activity. **c** pH, and **d** temperature stability of the *Gv*Abf. The residual activity was monitored, and the maximum activity was defined as 100%. Values shown were the mean of the duplicate experiments

Table 1 Effect of metal ions and chemicals on the enzymatic activity of *Gv*Abf

Ions[a]/chemical reagents[b]	Residual activity (%)
None	100
Na$^+$	101 ± 0.0
K$^+$	116 ± 1.0
Cd^{2+}	106 ± 0.3
Ca^{2+}	110 ± 0.0
Ni^{2+}	114 ± 1.5
Cu^{2+}	117 ± 1.3
Mg^{2+}	104 ± 1.6
Mn^{2+}	189 ± 2.2
Co^{2+}	120 ± 1.9
Zn^{2+}	57 ± 0.7
DTT	113 ± 0.0
EDTA	108 ± 1.3
Hexane	86 ± 0.3
EtOAc	120 ± 0.6
EtOH	95 ± 0.9
MeOH	86 ± 0.2
Acetone	100 ± 0.9
Acetonitrile	85 ± 0.0
BME	101 ± 0.6

[a]Final concentration was 1 mM

[b]Final concentrations for DTT and EDTA were 1 mM whereas the others were 1%. The standard deviations were calculated from from at least duplicate experiments

full activity at the optimum working conditions (pH 6.0 and at 75 °C after 12 h of incubation) [31]. In addition, α-L-Arabinofuranosidases from *C. saccharolyticus* and *C. thermocellum* have similar optimum temperature values, 80 and 82 °C, respectively [39, 40]. *C. saccharolyticus* has optimum pH 5.5 [39], which is nearly the same optimum working pH as *Gv*Abf. Interestingly, α-L-Arabinofuranosidase from *G. stearothermophilus* T-6 has the same optimum working temperature [30] as *Gv*Abf which correlates with the above mentioned sequence identity. α-L-Arabinofuranosidases from *T. maritima* and *T. petrophila* have different optimum temperature values as 90 and 70 °C and exhibited the optimum working pH as 7 and 6, respectively [41, 42]. Another α-L-Arabinofuranosidase from *T. xylanilyticus* shows 75 °C as optimum temperature. In summary, it was concluded that α-L-Arabinofuranosidases from gram positive bacteria have weak acid optimal Ph, while those from gram negative have slightly higher optimal pH.

The activity of *Gv*Abf was measured under standard assay conditions in presence of several metal ions and chemicals. Different effects on the *Gv*Abf activity was observed in the presence of various ions and chemicals on the hydrolysis of *p*NP-α-L-arabinofuranoside. Effects of metal ions, chemicals and organic solvents on *Gv*Abf activity were listed in Table 1. The enzyme activity was highly stimulated in the presence of 1 mM Mn^{2+}, Co^{2+} and 1% EtOAc (v/v). Similar stimulating effect was observed on α-L-Arabinofuranosidase activity from *Geobacillus Caldoxylolyticus* TK4 with Mn^{2+} and Co^{2+} ions [31]. Similarly, 1 mM Mn^{2+} ions had small stimulating effect on the activity of α-L-Arabinofuranosidase activity from *Bacillus*

Table 2 Characteristics of α-L-Arabinofuranosidase from *Geobacillus vulcani* GS90 and other microorganisms

Strain	K_m (mM)[a]	V_{max} (U/mg)[a]	K_m (mM)[b]	V_{max} (U/mg)[b]	Optimal temperature (°C)	Reference
Geobacillus vulcani	0.2	200	0.1	526	70	This study
Ruminiclostridium thermocellum	0.25	ND	ND	ND	82	X
Caldicellulosiruptor saccharolyticus	1.29	ND	ND	ND	80	W
Geobacillus caldoxylolyticus Tk4	0.17	588	ND	ND	75–80	T
Bacillus Stearothermophilus T-6	0.42	749	ND	ND	70	Y
Thermotoga maritima	0.42	ND	ND	ND	90	V
Thermotoga petrophila	ND	ND	19.9	478	73	Z

ND not determined

X: Taylor et al. [40]

W: Lim et al. [39]

Y: Gilead and Shoham [30]

V: Miyazaki [41]

T: Canakci et al. [31]

Z: Squina et al. [43]

[a]*p*NP-α-L-arabinofuranoside

[b]Sugar beet arabinan

stearothermophilus T-6 [30], endoarabinanase from *Thermotoga thermarum* [25] and α-L-Arabinofuranosidase from *Paenibacillus* sp. DG-22 [24]. However, no effect on activity of alpha-L-Arabinofuranosidase from *Anoxybacillus kestanbolensis* AC26Sari was detected in presence of 1 mM Mn^{2+}, Co^{2+} [22]. 1 mM Ca^{2+}, K^+, Cu^{2+}, Ni^{2+} and DTT had slight stimulating effect on the activity of enzyme. Addition of 1 mM Na^+, Cd^{2+}, Mg^{2+}, EDTA and 1% BME was not significantly changed the enzyme activity. Since the addition of EDTA did not affect the enzyme activity significantly, this result suggests that no metals are needed for enzymatic activity [24, 30]. Furthermore, 1 mM Zn^{2+} has inhibitory effect on the enzyme activity displaying 57% residual activity. Similar results were obtained with α-L-Arabinofuranosidase from *Paenibacillus* sp. DG-22 [24]. Interestingly, Zn^{2+} ions had no detected effect on α-L-Arabinofuranosidase activity from *G. stearothermophilus* T-6 [30]. However, in the presence of Mn^{2+} ion as well as Cu^{2+}, Zn^{2+}, and Ni^{2+} had a significant decrease on alpha-L-Arabinofuranosidase activity from *Thermotoga maritima* Msb8 [41] and GH43 endoarabinanase from *Thermotoga thermarum* [25]. Most of the solvents tested; acetone, hexane, ethanol, methanol and acetonitrile did not affect the hydrolysis of the substrate significantly.

Kinetic parameters of *Gv*Abf were determined with two different substrates; *p*NP-α-L-arabinofuranoside and sugar beet arabinan (Table 2). The *Gv*Abf exhibited a simple Michaelis–Menten kinetics and Lineweaver–Burk plot showed a linear response over the tested concentration range in both cases. *Gv*Abf has a higher affinity to *p*NP-α-L-arabinofuranoside and sugar beet arabinan compared to the other α-L-Arabinofuranosidases from GH51 superfamily with the exception of the one from *G. caldoxylolyticus* TK4 [31]. Regarding the K_m values with sugar beet arabinan, *Gv*Abf has about 200-fold higher affinity than α-L-Arabinofuranosidase from *Thermotoga petrophila* [43], showing the specificity of *Gv*Abf to its natural substrate. Interestingly, α-L-Arabinofuranosidase from the host *G. stearothermophilus* had twofold higher K_m value with *p*NP-α-L-arabinofuranoside [30], comparing to *Gv*Abf.

To assure GvAbf hydrolysis activity on its substrate, the products of sugar beet arabinan as a results of treatment with *Gv*Abf enzyme were analyzed via thin layer chromatography (TLC) (data not shown). TLC analysis indicated that arabinose was the only product to be released from sugar beet arabinan by the enzyme. Non treated arabinan with GvAbf did not give any product spots detected by TLC as expected. This result indicated that *Gv*Abf enzyme is an exo-acting enzyme that hydrolyzes the nonreducing end of its substrate. The finding here was consistent with other previously reported Abfs hydrolysis activity [22, 26, 31].

Synergistic action of *Gv*Abf in fruit juice enrichment

Polysaccharides and hemicellulosic components are responsible for turbidity and viscosity of the raw juices; therefore, carbohydrate and hemicellulose degrading enzymes have been exploited recently for extraction and enrichment of fruit juices in terms of yield, clarification and quality characteristics of juices [44]. Abfs are also known as accessory enzymes working with other xylanolytic enzymes together via an orchestral action. It is well know that Abf has a higher synergistic impact especially with xylanase rather than individual Abf action [4–8]. Importantly, Abf and other hemicellulases like xylanase play synergistically a promising role in the beverage industry [5, 45].

In the present study, the synergistic role of the *Gv*Abf was investigated in fruit juice enrichments of apple, grape, peach and orange pulps. For this purpose, a commercially available xylanase enzyme was also used in our studies. The effect of xylanase has been previously demonstrated in many studies for fruit juice extraction and clarification process [17, 36, 46–48]. Therefore, the clarity level, extraction yield and the reducing sugar content of variety fruit juices were tested applying *Gv*Abf and xylanase in separate and combined manner in this study.

Treatment with *Gv*Abf itself led to increase of 10.93, 2.94, 10.16 and 7.27% in the yield of apple, grape, peach and orange juices, respectively, compared to control untreated juice (Table 3). Obviously, commercial xylanase displayed better yield compared to *Gv*Abf alone. The best results were obtained with simultaneous treatment of both enzymes with 26.56, 30.88, 32.20 and 40.00% increase in the yield of apple, grape, peach and orange juices, respectively, compared to untreated juice as control. The increase in the yield may be correlated with the breakdown of all polymeric carbohydrates, hemicelluloses and starches of the pulp, thus increasing the yield of juice by enabling better processing [44]. Similar results have been achieved in presence of xylanase enzyme for increasing yield of fruit juices and the maximum apple juice yield is obtained as 15.35% in presence of xylanase from *Streptomyces* sp. [17]. So far, there is no any results has been reported with any Abfs in terms of increasing fruit juice yield in the literature.

The juice clarity, measured in terms of % transmittance (%*T*) at 660 nm, was also significantly affected by increase of 47.26, 25.99, 44.97 and 41.77 %*T* in apple, grape, peach and orange juice, respectively, in presence of both *Gv*Abf and xylanase enzymes (Table 3). The efficiency of *Gv*Abf in clarity of all treated fruit juices was very low (3.49 for grape, 10.88 for orange, 5.50% for peach and 14.36% for apple); however, it displayed additive effect with the xylanase enzyme together. The effect of xylanase itself was better compared to *Gv*Abf treatment for the clarity of all tested juices in our studies. Several reports are available

Table 3 Effect of *Gv*Abf, xylanase and *Gv*Abf + xylanase treatment on fruit juice clarification, reducing sugar content and yield

Fruit juices	Grape	Orange	Peach	Apple
Clarity (%*T*)				
Control	66.11 ± 0.2	51.56 ± 1.1	47.27 ± 0.4	58.33 ± 2.7
*Gv*Abf	68.42 ± 1.7	57.17 ± 0.8	49.88 ± 0.3	66.71 ± 0.6
Xyl	79.12 ± 3.2	68.03 ± 1.9	59.07 ± 0.9	71.14 ± 2.0
*Gv*Abf + Xyl	83.29 ± 1.9	73.10 ± 0.7	68.53 ± 1.3	85.90 ± 1.5
Reducing sugar (μg/ml)				
Control	655.01 ± 2.3	361.71 ± 1.8	850.21 ± 2.6	917.13 ± 4.3
*Gv*Abf	698.07 ± 4.1	369.43 ± 4.1	857.93 ± 3.3	945.55 ± 2.9
Xyl	751.68 ± 3.9	387.18 ± 5.2	885.22 ± 3.9	1007.01 ± 1.7
*Gv*Abf + Xyl	765.89 ± 3.7	401.07 ± 4.4	893.66 ± 3.0	1151.19 ± 5.5
Yield (ml)				
Control	68 ± 0.1	55 ± 1.6	59 ± 0.7	64 ± 1.3
*Gv*Abf	70 ± 0.3	59 ± 0.8	65 ± 0.4	71 ± 0.7
Xyl	84 ± 1.4	71 ± 0.3	73 ± 1.6	73 ± 0.6
*Gv*Abf + Xyl	89 ± 1.2	77 ± 0.9	78 ± 0.5	81 ± 0.9

The weight of each fruit used for the experiment was 100 g. Control means that no enzyme in the test

on the use of pectinase and other enzymes for clarification of fruit juice but literature citing the use of xylanases for the clarification of fruit juice is hardly available [17, 48] and there is no any study with Abfs for the same purpose so far.

The reducing sugar content in the all enzyme treated and extracted juices in our studies was also found to be more than control juices (Table 3). The maximum increase of reducing sugar level was observed in the case of grape juice among all *Gv*Abf treated samples with an increase of 6.57% (apple was 3.10%, orange was 2.13% and peach was 0.91%). The effect of xylanase on peach juice displayed better increase in terms of reducing sugar level (41.17%) compared to untreated control and it was followed by grape (14.75%), orange (7.04%), and apple (9.80%) in our studies. Even though the highest reducing sugar level was observed in apple juice (1151.19 μg/ml), the maximum increase of reducing sugar level was achieved with peach juice (%51.10) in simultaneously treatment of *Gv*Abf and xyalanse. The similar studies in terms of release in reducing sugar level were obtained with xylanase enzyme for the application of fruit juices [17, 36, 46]. Similarly, the release of reducing sugar contents of apple, grape and orange juices were improved by 17.21, 16.79, and 19.57%, respectively, with xylanase from *Streptomyces* sp. [17]. In another study, addition of xylanase from *Geobacillus* sp. TF16 to apple and orange juices increased the reducing sugar content 1.03 and 2.85-fold, respectively [46]. Xylanase from *Sclerotinia sclerotiorum* S2 fungus also improved the reducing sugar level of orange and apple juice 5 and 10 times, respectively after 24 h treatment [36]. Up to date, there is no any study related to reducing sugar level of fruit juices treated with any Abf enzyme.

Conclusions

In this study, for the first time, a thermostable α-L-Arabinofuranosidase (*Gv*Abf) from previously identified *G. vulcani* GS90 by 16S rRNA was cloned in *E. coli* DH5α and expressed in *E. coli* BL21 (λDE). The biochemical properties were obtained in terms of utilization of this enzyme as a potential candidate in variety of industry. Well known synergistic role with xylanase enzymes also observed in the fruit juice extraction and clarification process. *Gv*Abf obtained from *G. vulcani* GS90 could be remarkably helpful in facilitating industrial application as an accessory enzyme, especially in fruit juice enrichment processes.

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