Industrial Applications of Endoglucanase Obtained from Novel and Native *Trichoderma atroviride*

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An endo-β-1,4-glucanase (EG), produced under submerged fermentation by local isolate *Trichoderma atroviride*, was purified using ammonium sulfate precipitation, column and ion exchange chromatography with 55.16 fold and a specific activity of 30.9 EU mg^{-1}. The studies of PAGE, SDS-PAGE and zymogram test have been carried out. The EG had optimum activity at pH 5.0 and 50 °C respectively. Using CMC as substrate, the enzyme showed maximum activity (V_{max}) of 6.7 (μmol glucose min^{-1}) mL^{-1} with its corresponding K_m (Michaelis-Menten constant) value of 1.12 mg mL^{-1}. While the EG activity was activated by NaCl, inhibited by $MgCl_2$ and $MgSO_4$. Otherwise, Tween 80, Triton X-100 and the total saponins known as biosurfactants enhanced the EG activity. The obtained EG had low K_m and high thermal stability. Additionally, usability of the EG in biotechnological application was investigated. The results showed that the EG had potentially sufficient effects on denim fabric and pretreated lignocellulosic wastes.

Key words:

endo-β-1,4-glucanase, purification, characterization, biosurfactant, saponin, *Trichoderma atroviride*

Introduction

Lignocellulosic materials are cheap and the most abundant material on Earth. Especially, agricultural wastes (e.g. wheat straw and corn stover), forestry residues (e.g. mill wastes and sawdust), portions of municipal solid waste, and most industrial wastes are their source.[1]

Bioconversion of cellulose to soluble sugar is performed by cellulases.[2] For effective degradation of cellulose, a complete enzyme system is necessary, which comprises three classes of enzymes; endo-β-1,4-glucanase (CMCase, EC 3.2.1.4, EG; randomly cleaving β-linked bonds within the cellulose molecule), cellobiohydrolases (EC 3.2.1.91, CBH; removing cellobiose units from nonreducing ends of the cellulose chain) and β-glucosidase (cellobiase, EC 3.2.1.21, hydrolyzing cellobiose and cellooligosaccharides to glucose).[3,4,5] These cellulases can be produced by most microorganism, including fungi, bacteria, and actinomycetes. *Trichoderma* and *Aspergillus* species are the most studied cellulolytic microorganisms for cellulase production.[6]

Lignocellulosic materials mainly consist of cellulose, hemicelluloses, and lignin. Cellulose is the main component of lignocellulosic materials, but there are two major obstacles for hydrolysis of cellulose in lignocellulosic material. These are the recalcitrance of crystalline cellulose itself, and the highly protective lignin surrounding it. These problems can be eliminated using pretreatment method. Pretreatment methods provide increasing cellulose accessibility for enzymatic hydrolysis.[7,8]

Cellulases are the most important enzymes used in various industrial applications, such as food, textiles and laundry, pulp and paper, and agricultural, bioethanol production from lignocellulosic waste, extraction of fruit and vegetable juices, alongside research purposes.[9] For this reason, there is an increasing demand for obtaining new, more specific, stable enzymes and the screening and characterization of novel isolates.[10,11]

Denim is remarkably popular in the textile industry. To create diversity in denim fabric, it is necessary to find new convenient applications to respond the huge market demand. Biostoning is the most successful textile application of cellulases. This application is used in denim finishing, creating the old-look and worn look in denim garments. The old look appearance in denim garments using cellulases results from non-homogeneous removal of indigo dye trapped inside the fibers. Cellulase treatment of denim fabric is an environment-friendly method compared to with stone washing.[12,13]

Otherwise, cellulases are utilized to improve the appearance of cellulosic garments by removing fuzz

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fibers and pills from the surface. Because the amorphous region of cellulose causes fuzz and pill formation on the garment surface, using a single type cellulase, preferentially endoglucanase, will be sufficient for removing fuzz and pill of amorphous cellulose.[13]

The purpose of this study was to determine some properties (optimum pH, temperature, thermal stability, and kinetic properties) of EG from a local isolate *Trichoderma atroviride*. Otherwise, the paper presents the effect of EG on the properties of pretreated lignocellulosic wastes and denim fabric.

Materials and methods

Chemicals

Carboxymethylcellulose sodium salt (CMC-Na), 3,5-dinitrosalicylic acid (DNS) was obtained from Alfa Aesar (USA), Sephadex G-100, DEAE- Sephadex was obtained from Sigma Chemicals Co. Ltd (USA). Other chemical reagents were analytical grade.

Microorganism and plants

The microorganism used as EG producer was isolated from the shore of hot spring water, Karahayıt, Denizli. In this study, DNA isolation was performed using the classical phenol-chloroform method. Purity and concentration measurements were determined using the nanodrop spectrophotometer (Thermo). The polymerase chain reaction (PCR) was carried out at 95 °C for 5 minutes, 35 cycles of 1 minute at 94 °C, 1 minute at 55 °C, 1 minute at 72 °C, plus an additional 7 minute cycle at 72 °C. Internal transcribed spacer (ITS) primers were used in the reactions (ITS1 and ITS4). Electrophoresis was conducted in agarose gel (1.2 %). For sequencing, the sample was sent to Macrogen Europe. Sequence data was processed with Blastn. Sequence alignment was performed by using MEGA 6.06 software. The strain used in this study was a member of the genus *Trichoderma*, and identified as *Trichoderma atroviride*.

The *Gypsophila arrostii* Guss. var. *nebulosa* (Boiss. & Heldr.), *Gypsophila pilulifera* Boiss. & Heldr. and *Ankyropetalum reuteri Fenzl.* Boiss. were collected from Isparta, Antalya and Adıyaman Provinces of Turkey, respectively, and identified by the Department of Biology, Suleyman Demirel University.

The roots of the plants were dried to constant weight and then pulverized. Initially, the dried root powder for each plant (100 g) was extracted by stirring the suspension in petroleum ether as an apolar solvent at room temperature. Secondly, the saponins from the plant powder were extracted eight times with 70 % aqueous ethanol solution. The solvent was evaporated at 100 mbar, 80 °C, and the extract solution was then concentrated to remove the solvent under vacuum. After drying, total saponins for the *Gypsophila arrostii* (Total saponin I), *Gypsophila pilulifera* (Total saponin II) and *Ankyropetalum reuteri* (Total saponin III) (18 g, 13.5 g and 16 g, respectively) were obtained.

Cultivation of *T. atroviride*

T. atroviride was cultured in a medium as described previously.[14] 1 % (w/v) CMC was used as carbon source. Initial pH of the medium was adjusted at 5.0 with HCl and the medium was sterilized by autoclaving at 121 °C for 15 minutes. After the culture medium was inoculated with *T. atroviride*, it was incubated at 35 °C with rotary shaking at 120 rpm. The EG activity was measured for 6 days. A sample was withdrawn in medium every 24 h and centrifuged at 8.000 rpm for 30 minutes. The EG activity of supernatant was determined.

Endo-β-1,4-glucanase activity assay

The endo-β-1,4-glucanase activity was determined based on the Ghose method[15], and reducing sugars were measured using the 3,5 Dinitrosalicylic acid (DNS) method.[16] The amount of reducing sugars liberated from CMC solubilized in 50 mM Na-citrate buffer (pH 5.0) by the enzyme was determined in this assay. The reaction was performed at 50 °C, pH 5.0 for 30 minutes. One unit of endo-β-1,4-glucanase activity was defined as the amount of enzyme that could hydrolyze CMC and release 1 μmol of glucose within 1 minute of reaction. The optical absorbance was measured at 540 nm.[15] The cellulase activity was determined by using a calibration curve for glucose.

Purification of EG

After five days of cultivation at 35 °C with shaking (120 rpm), the culture medium was centrifuged at 8.000 rpm for 30 minutes and the supernatant was used for purification of EG as the crude enzyme solution. All procedures were carried out at 25 °C.

Solid ammonium sulfate was added to the supernatant at 60–80 % saturation, and precipitated proteins were collected by centrifugation (8.000 rpm, 30 minutes). The proteins were redissolved in a small amount of 50 mM Na-citrate buffer (pH 5.0) and then dialyzed against the same buffer. The dialyzed EG solution was then applied onto a pre-equilibrated Sephadex G-100 column (1x30 cm) and elution was performed with 50 mM Na-citrate buffer (pH 5.0) at 1 mL min^{-1}. Fractions containing EG activity were pooled and loaded onto DEAE Sepha-

dex column (1x10 cm). Elution was carried out with 50 mM Na-citrate buffer (pH 5.0) containing a linear gradient of NaCl from 0–0.5 M at 1 mL min^{-1}. The active fractions were pooled and then dialyzed against 50 mM Na-citrate buffer (pH 5.0). The obtained preparation was concentrated with lyophilization, and then used for kinetic studies and applied to SDS polyacrylamide gel electrophoresis (SDS-PAGE) and native polyacrylamide gel.

Native polyacrylamide gel electrophoresis (Native-PAGE) and zymogram test

A 10 % native PAGE separation gel containing 0.2 % (w/v) CMC was prepared for the zymogram test. Electrophoresis was performed at room temperature for 2.5 h at 150 V using Scie Plas TV100 Twin-Plate (10 x 10 cm). After native PAGE, the gel was cut into two pieces for EG activity staining. One of the pieces was stained using the silver staining method. After other piece of the gel was incubated in Na-citrate buffer (pH 5.0) at 50 °C for 30 minutes, stained for 10 minutes with 0.1 % Congo red, and then washed with 1 M NaCl, until the enzyme bands had become visible.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

This study was performed according to the method of Laemmli (1970).[17] An equal volume of sample buffer (10 % glycerol, 5 % β-mercaptoethanol, 2 % SDS, and 0.05 % bromophenol blue in 0.25 M Tris-HCl buffer; pH 6.8) was added to the purified enzyme and incubated in boiling water for 2 minutes. The samples were then subjected to SDS-PAGE (resolving gel and stacking gel, 10 % and 5 %, respectively) using Scie Plas TV100 Twin-Plate (10 × 10 cm). Electrophoresis was performed at room temperature for 2 h at 150 V. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250. Thermo Scientific PageRuler™ plus prestained protein ladder was used as standard marker.

Determination of the protein concentration

Protein concentration was determined using the Lowry method.[18] Bovine serum albumin (BSA) was used as standard protein.

Optimum pH and temperature

The optimum pH for endo-β-1,4-glucanase activity was determined using different pH buffers: 50 mM Na-citrate buffers (pH 4.0–5.0), and potassium phosphate buffers (pH 6.0–9.0). The optimum temperature for endo-β-1,4-glucanase activity was determined under standard assay conditions at temperatures from 30 °C to 70 °C.

Thermal stability

Thermal stability studies were carried out by incubating the enzyme at 50 °C for various durations (between 30 minutes–72 h). The amount of released reducing sugars was then determined by the DNS method.

Determination of kinetic parameters

For determining Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) of the purified enzyme, the enzyme activity was detected using different concentrations of CMC (0.7–2.63 mg mL^{-1}) in 50 mM Na-citrate buffer (pH 5.0). The K_m and V_{max} were calculated from Lineweaver Burk plot.

Substrate specificity

Hydrolytic activity of purified EG toward 1 % (w/v) Avicel, cellulose, cellobiose, CMC, filter paper, and wheat bran (WB) in 50 mM Na-citrate buffer (pH 5.0) was determined to evaluate the substrate specificity. The enzyme activity based on the amount of released reducing sugars was determined by the DNS method.

Effect of some metal ions on the enzyme activity

The effects of metal ions on the EG were determined by adding different concentrations of NaCl, $MgCl_2$, KCl, $CoCl_2$, $CuCl_2$, $BaCl_2$, $CaCl_2$, $FeCl_3$, $MnSO_4$, $FeSO_4$, $CuSO_4$, and $MgSO_4$ to the activity assay medium.

Effects of surfactants and biosurfactants on the enzyme activity

The effect of surfactants and biosurfactants on the EG was determined by adding different concentrations of Triton X-100, Tween 80, total saponins to the activity assay medium. The effect of Tween 80 and Triton X-100 on EG was studied between 0.01 w/v– 0.04 w/v and 0.0018 w/v– 0.04 w/v, respectively. Concentration of *Gypsophila arrostii* (Total saponin I), *Gypsophila pilulifera* (Total saponin II) and *Ankyropetalum reuteri* (Total saponin III) were 0.02–0.09, 0.01–0.09, 0.02–0.07 mg mL^{-1}, respectively.

Effect of the EG on the pretreated lignocellulosic waste

Sawdust of calabrian pine, abies pine, poplar and WB were provided by Faculty of Forestry, Suleyman Demirel University.

Pretreatment of sawdust and WB was performed using 0.5 and 1 M of NaOH and 0.05 and 0.1 M H_2SO_4. In 1 L of these solutions, 100 g of waste was mixed and incubated at 1 h at 72 °C.[19]

The waste was then filtered, washed using tap water and distilled water until neutralization (pH 7.0), and dried in a drying oven at room temperature. Additionally, the pretreatment without chemical addition was carried out with distilled water and used as control.

Enzymatic hydrolysis was carried out in a tube, with 2 % w/v concentration of pretreated lignocellulosic waste in 0.05 M Na-citrate buffer (pH 5.0) at 50 °C for 4 h. After enzymatic hydrolysis, it was centrifuged, and the supernatant was used to determine the amount of reducing sugars released from the pretreated lignocellulosic waste. The sample without enzyme was used as blank.

After lignocellulosic waste treatment with EG, the amount of reducing sugars (mg g^{-1}) was determined by using the DNS method. The experiments were repeated three times. The results were evaluated using OriginPro 8.5 program by one-way Analysis of Variance (ANOVA) at a 5 % significance level, and then Tukey's test was applied to the averages.

Treatment of denim fabric with cellulase

Desized cotton denim fabric from Bossa (TR) was used in this study. The cotton denim fabric (2x2 cm) was treated with enzyme at pH 5.0 and 50 °C for 24 h. Another piece of cotton denim fabric (2x2 cm) was treated without enzyme addition under same conditions as control. Enzyme activity was halted by incubating the fabric for 10 minutes in 80 °C water, and then washed in warm and cold water and dried at room temperature.

Scanning electron microscopy (SEM, a Tescan Vega-LSU instrument) was used to assess the surface morphology of the treated and untreated denim fabric. The micrographs were taken at X1 and X100 magnification level.

Results and discussion

Cultivation of *T. atroviride*

Isolated fungi was identified through molecular characterization. BLAST search was performed using alignment of partial *18S rRNA* gene (Fig. 1) isolated from fungi. Herewith, it exhibited maximum homology (99 %) with *T. atroviride* strain wxm143 (accession number: HM047763.1).

Fungi are favorable enzyme-producers among microbes because they are able to produce production of extracellular enzymes which are easy to recover from the fermentation broth. In this study, *T. atroviride* was used as EG-producer in a submerged system. Previously, Grigorevski-Lima *et al*.[20] used the wild strain of *T. atroviride* in their study, and identified it as a promising producer of lignocellulolytic enzymes (EG, FPase, xylanase, β-glucosidase). Although submerged fermentation can lead to product dilution and decreasing of enzyme stability, it is advantageous in terms of process control and easy recovery of extracellular enzymes, mycelia or spores.[21] In previous studies, most of the enzyme production from *T. atroviride* had been performed using submerged fermentation.[20,22,23,24] Moreover, submerged fermentation is still a prevalent method for cellulase production by fungi.[25]

The EG production was carried out at 35 °C, pH 5.0 for six days. The EG activity was determined in culture medium from the second to sixth day of fermentation. It was determined that EG production reached maximum (1.75 EU mL^{-1}) on the fifth day of fermentation (Fig. 2). After that, the enzyme activity decreased. There are several studies on the production of EG using different carbon sources. Oliveira *et al*. obtained EG activity of 1.37 EU mL^{-1} and 1.59 EU mL^{-1} using *T. atroviride* 679 and *T. atroviride* NTG21, respectively.[24] Grigorevski-Lima *et al.* determined the maximum EG activity for *T. atroviride* 679 using untreated sugarcane bagasse as 1.9 EU mL^{-1}.[20] Odeniyi *et al.* determined that EG activity was 0.096 EU (1.6 nkat) in submerged fermentation.[26]

Purification of EG

After removal of the cell from the culture medium, the EG was purified from supernatant obtained by ammonium sulfate precipitation, Sephadex G-100 and DEAE-Sephadex column. The

> Trichoderma atroviride

ATTTCCATGGTCATACGTGCAGTGATCGAGGTCACATTTCAGAAGTTGGGTGTTTAACGGCTGTGGACGCGCC
GCGCTCCCGATGCGAGTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCGAGACCGCCACTGTATTTCGGAGA
CGGCCACCCGCTAAGGGAGGGCCGATCCCCAACGCCGACCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCT
CGGACAGGCATGCCCGCCAGAATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTG
CAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTT
TGATTCATTTTCGAAACGCCTACGAGAGGCGCCGAGAAGGCTCAGATTATAAAAAAACCCGCGAGGGGGTAT
ACAATAAGAGTTTTAGGTTGGTCCTCCGGCGGGCGCCTTGGTCCGGGGCTGCGACGCACCCGGGGCAGAGAT
CCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGGGAGTTGTAAACTCGGTAATGATCCCTCCGCA
GGTACCCCTACCGGAAGGGTTACCAAAAATTTCCT

Fig. 1 – *Alignment of partial 18S rRNA gene isolated from T. atroviride*

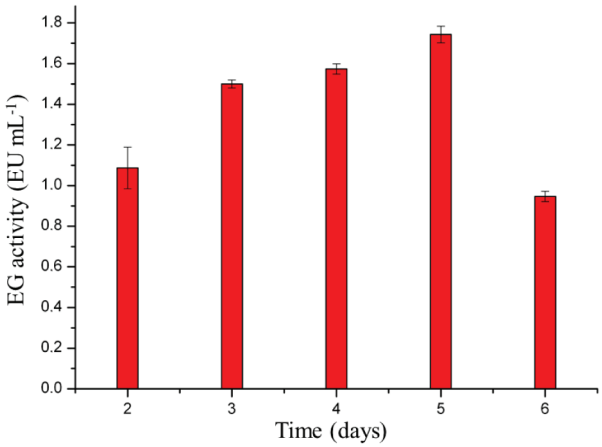


Fig. 2 – *Determination of optimum day for enzyme production from T. atroviride. Each value represents the mean ±SD for three determinations.*

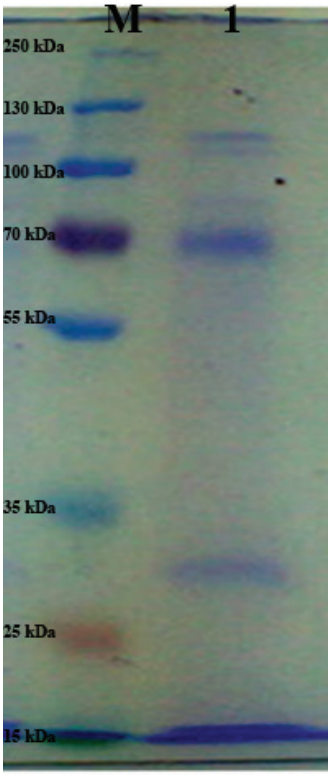


Fig. 3 – *SDS PAGE bands of EG produced by T. atroviride. M. Marker; 250 kDa, 130 kDa, 100 kDa, 70 kDa (pink band), 55 kDa, 35 kDa, 25kDa (orange band), 15 kDa (green band).*

specific activity of purified enzyme was 30.9 EU mg^{-1} and a yield of 0.35 %. The purification procedure is summarized in Table 1. After purification, the obtained enzyme solution was concentrated 10-fold with lyophilization before native PAGE and SDS-PAGE.

The specific activity of EG from different types of microorganisms was examined from the Comprehensive Enzyme Information System.[27] The specific activity values of EG (EU mg^{-1}) changed from 0.018 (*Thermobifida fusca*) to 4240 (*Streptomyces ruber*). This value was 0.258 EU mg^{-1} for *Trichoderma viride* and 68 EU mg^{-1} for *Trichoderma longibrachiatum*.[27] Usage of different kinds of substrate can effect specific activity.[28]

While the purified and lyophilized enzyme appeared as six bands in SDS-PAGE (Fig. 3), it appeared as three bands in native-PAGE (Fig. 4b) and the two of them had shown EG activity in the zymogram (Fig. 4a).

Earlier, Coral *et al*.[29] had reported that there were two bands in SDS PAGE of CMCase from *Aspergillus niger* Z10. Kumar *et al*.[30] identified two bands of CMCase weighted at 34 and 26.5 kDa in SDS PAGE, and explained that cellulase is a heteromeric multienzyme complex, and these might correspond to domains belonging to the purified enzyme in SDS-PAGE.[30]

Optimum pH and temperature

At pH 5.0, EG activities were measured at various temperatures from 30 °C to 70 °C in order to determine the optimum temperature. EG showed maximum enzyme activity at 50 °C (Fig. 5a). Enzymes can be classified into three groups, hyperthermophilic (>80 °C), thermophilic (50 °C – 80 °C), and mesophilic (40 °C – 60 °C) depending on the optimum temperature. It is proposed that fungi-obtained endoglucanase has mesophilic properties.[5]

While the cellulases from *A. terreus* DSM 826 and *A. glaucus* XC9 have optimum temperature (50 °C) similar to our enzyme[3,5], the EG from *T. atroviride* 676 has higher optimum temperature.[20] Tao *et al*. had indicated that *T. reesei*, the most popular industrial cellulase producer, has optimum temperature at 52 °C.[5]

Table 1 - *Summary of the purification of endo-β-1,4-glucanase from T. atroviride*

Step	Total protein (mg)	Total activity (EU)	Specific activity (Eu mg^{-1})	Yield (%)	Purification fold
supernatant	593.4	1066.05	0.56	100	1
ammonium sulfate precipitation (60 – 80 %)	15.75	2.94	5.36	2.65	9.57
column chromatography	3.13	0.26	12.04	0.53	21.5
ion exchange chromatography	2.07	0.07	30.9	0.35	55.16

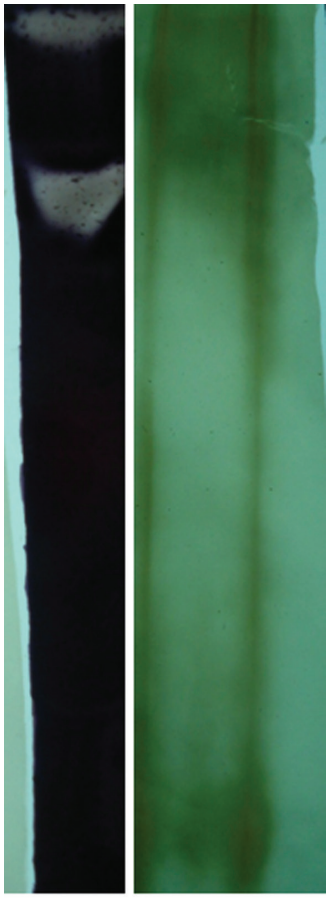


Fig. 4 – *(a) Zymogram test of EG (b) Native PAGE of EG*

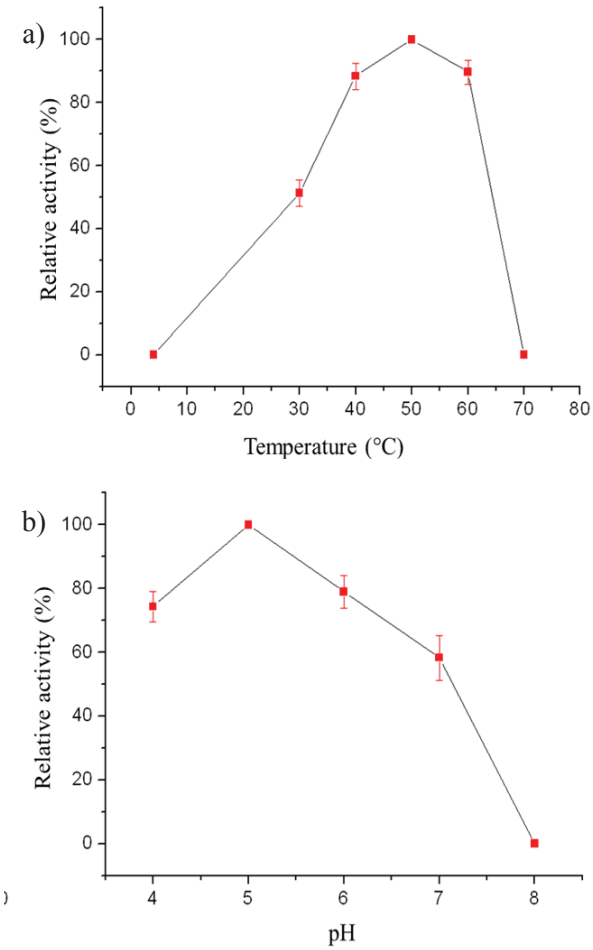


Fig. 5 – *Effect of temperature (a) and pH (b) on the EG. Each value represents the mean ±SD for three determinations.*

The optimum pH of EG activity for hydrolysis of CMC at 50 °C was determined. EG from *T. atroviride* showed maximum enzyme activity at pH 5.0 (Fig. 5b). Because the EG from *T. atroviride* showed optimum activity at pH 5.0, it can be said that the obtained enzyme has acidic properties according to Araŭjo *et al*.[31]

The optimum pH of this enzyme obtained from *T. atroviride* was same as that from *A. aculeatus*[32] and differed slightly from *T. atroviride* 676 (pH 4.0),[20] *T. reesei* QM-9414 (pH 4.5),[33] *A. terreus* DSM 826 (pH 4.8)[3], and *A. glaucus* XC9 (pH 4.0).[5]

Usage of acidic cellulases is more suitable for degradation of lignocellulosic materials, and biostoning and finishing of cellulosic fibers in the textile industry.[34, 35] It is suggested that this enzyme is convenient for potential industrial processes under conditions of optimum temperature and pH.

Thermal stability

High adsorption capacity, catalytic efficiency, and thermal stability are distinctive factors for cellulases in industrial applications.[35] Thermal stability of the EG was determined as described in materials and methods (at 50 °C, pH 5.0). The EG activity sustained up to 72 h, it lost only 9.5 % of its initial activity at the end of this time (Fig. 6). As shown in Table 6, EG from *T. atroviride* had better thermal stability in relation to most of the EGs from other fungi reported in previous studies.

Kinetic parameters

The effect of substrate concentration on EG rate for CMC was determined. The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were calculated from Lineweaver-Burk plots. K_m and V_{max} values of the EG for CMC hydrolysis were 1.12 mg mL^{-1} and 6.7 µmol glucose min^{-1} mL^{-1}, respectively (Fig. 7).

The obtained K_m value was relatively lower as compared to *Penicillium pinophilum*, *T. harzianum* ETS 323, and *A. glaucus* XC9 (Table 6). This suggested that the EG from *T. atroviride* showed good affinity towards CMC.

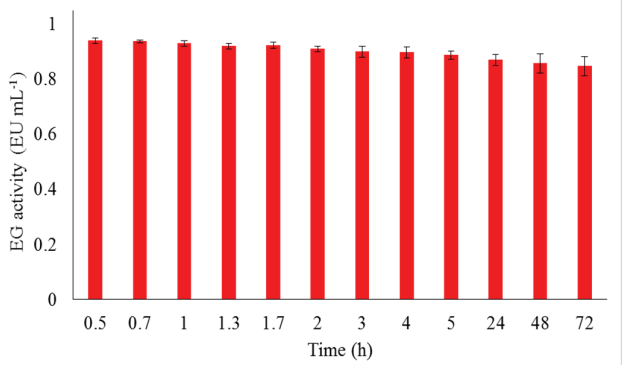


Fig. 6 – *Thermal stability of the EG (at 50 °C, pH 5.0). Each value represents the mean ±SD for three determinations.*

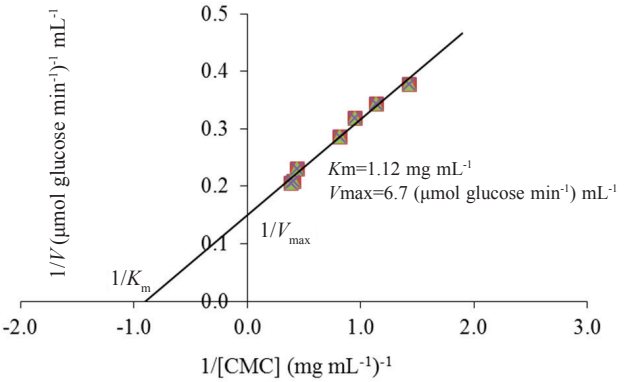


Fig. 7 – *Lineweaver-Burk graph for determination of K_m and V_{max} using CMC as substrate*

Substrate specificity

The substrate specificity of EG toward avicel, cellulose, cellobiose, wheat bran, filter paper, and CMC were determined. While the purified enzyme showed the highest activity against CMC, the activity toward avicel, cellulose, filter paper, and WB were lower (Table 2).

Effects of some metal ions on hydrolysis

The effect of metal ions on hydrolysis may be due to changes in the electrostatic bonding, which are responsible for changes in the conformation of the enzyme.[36, 37] Metal ions can cause either inhibition of enzyme or activation, based on the nature of the cations.[36] Determining the effect of metal ions on enzyme hydrolysis is important because many industrial applications require their addition at various stages of the process, and the greater part of the enzymes requires a specific metal ion as a cofactor for their catalytic activity.[36, 38]

Table 2 - *Activity for different substrate of T. atroviride EG*

Substrate	Specific activity (EU mg^{-1})
CMC	30.9
wheat bran	14.2
avicel	4.2
cellulose	3.8
filter paper	3.8

Experiments were conducted in the activity method described above with different concentrations of various salt ions. Effects of the metal ions on the enzyme activity for hydrolysis of CMC are shown in Table 3.

EG was able to retain 44 % and 47 % of the EG initial activity in the presence of 36.10^{-3} M $MgCl_2$ and 57.10^{-3} M $MgSO_4$, respectively (Fig. 8a and 8b). Inhibition effect of Mg^{2+} may be explained with the presence of at least one sulfhydryl group in the active site.[39] In previous studies, $MgCl_2$ and $MgSO_4$ inhibited the EG from *Bacillus cereus* and *A. terreus*, respectively.[3,63]

NaCl was studied between $3.5.10^{-3}$ M – $14.4.10^{-3}$ M (Fig. 8c). According to our results, the EG was activated with NaCl up to $14.4.10^{-3}$ M. It was reported that Na^+ had no effect on EG from *A. glaucus* XC9, but activated EG from *Cellulomanas* sp. YJ5.[5,40] Sinegani and Emtiazi[41] indicated that endoglucanase activity activated in the presence of Na^+.

Table 3 - *Effects of metal ions and surfactants on hydrolysis*

Chemicals	Relative activity (%)	Inhibition / activation
salt ions		
$MgCl_2$ ($36{\cdot}10^{-3}$ M)	44	inhibition
NaCl ($14.4{\cdot}10^{-3}$ M)	107	activation[a]
KCl	–	NE
$CoCl_2$	–	NE
$CuCl_2$	–	NE
$BaCl_2$	–	NE
$CaCl_2$	–	NE
$FeCl_3$	–	NE
$MgSO_4$ ($57 \cdot 10^{-3}$ M)	47	inhibition
$MnSO_4$	–	NE
$FeSO_4$	–	NE
$CuSO_4$	–	NE
surfactants		
tween 80 ($2 \cdot 10^{-2}$ w/v)	127	activation[b]
triton x-100 ($3.6 \cdot 10^{-3}$ w/v)	123	activation[c]
biosurfactants		
total saponin I (0.09 mg mL^{-1})	125	activation
total saponin II (0.09 mg mL^{-1})	116	activation
total saponin III (0.09 mg mL^{-1})	163	activation

[a]; up to $15 \cdot 10^{-3}$ M, [b]; up to 0.02 g mL^{-1}, [c]; up to 0.0036 g mL^{-1}, NE; no effect ($3.6 \cdot 10^{-3}$ M – $57 \cdot 10^{-3}$ M)

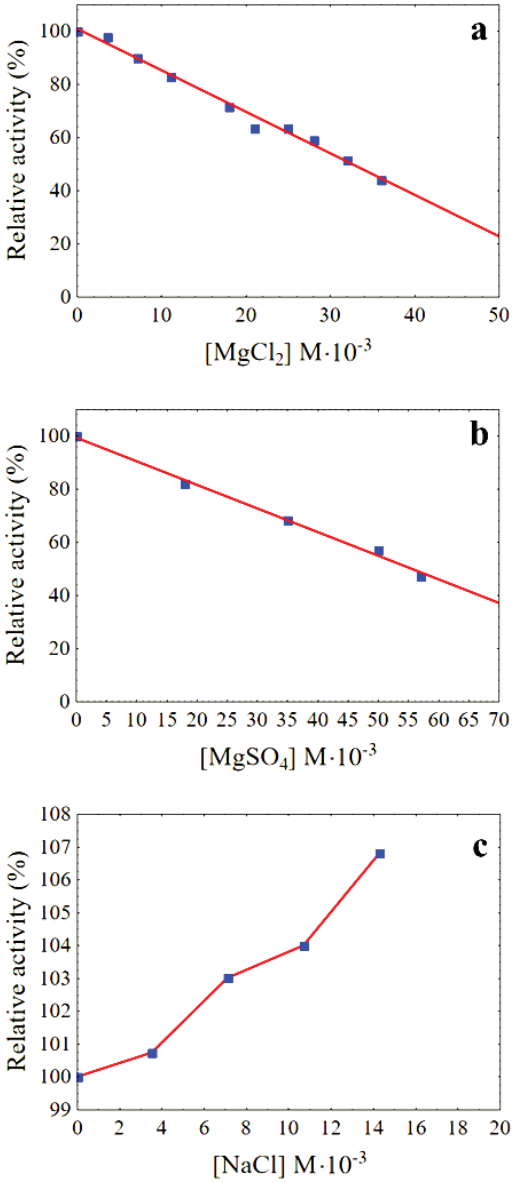


Fig. 8 – *Effects of $MgCl_2$ (a), $MgSO_4$ (b), and NaCl (c) on EG activity*

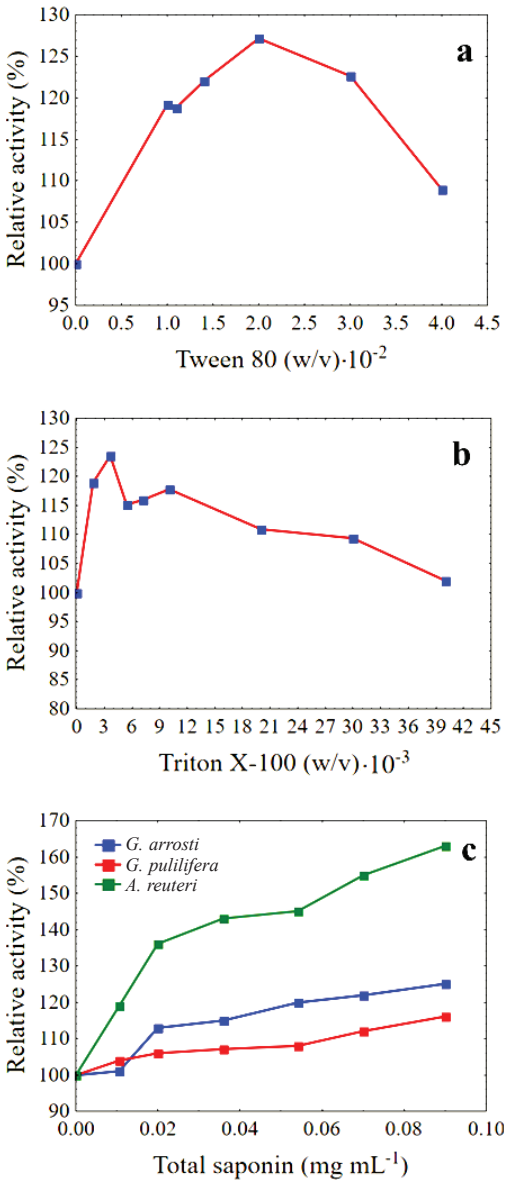


Fig. 9 – *Effects of Tween 80 (a), Triton X–100 (b), and total saponins as biosurfactants (c) on EG activity*

Salt ions prevent the enzyme from forming random hydrogen bonds and provide accessibility to active sites for substrate.[42]

Effects of surfactants and biosurfactants, total saponins on hydrolysis

EG activity was activated by increasing concentrations of Tween 80 and Triton X-100 (Table 3 and Fig. 9a, 9b).

It has been determined in previous studies that several surfactants such as nonionic Tween 20, 80, 81 and polyethylene glycol, cationic Q-86W, sophorolipid, rhamnolipid, and bacitracin had enhanced cellulose hydrolysis. The surfactant enabled creation of a hydrophilic environment on cellulose surface and thereby, it is provided that the irreversible binding of cellulase on cellulose decreased and enzyme activity increased.[43,44]

Saponins are glycosides with a distinctive foaming characteristic. They are found in many plants, the roots of which give soap-like foams in water. They consist of a polycyclic aglycone containing a choline steroid or triterpenoid.[45] The *Gypsophila arrostii* Guss. var. *nebulosa* (Boiss. & Heldr.) (Total saponin I), *Gypsophila pilulifera* Boiss. & Heldr. (Total saponin II), and *Ankyropetalum reuteri Fenzl.* Boiss. (Total saponin III), are typical Turkish varieties, naturally found in a particular area in the Mediterranean and Southeast zone of Turkey. These species are rich a source of triterpene saponins, and can be used to enhance activity of the EG (Table 3), which makes the process less economically feasible.

Fig. 9c shows that the total saponins had a beneficial effect on EG activity. When we compared chemical surfactants and biosurfactants as total saponin, it was observed that biosurfactants are more effective for increasing EG activity. Especially, *Ankyropetalum reuteri Fenzl.* Boiss. (Total saponin III) increased EG activity about 60 %. It has been reported in the previous studies that surfactants were the most suitable additives for improving the saccharification of lignocellulose and the recovery of cellulolytic.[46, 47]

Although studies have been reported on the effect of saponins from different plants on cellulose,[48,49] to our knowledge, no research paper is available on the effect of saponins from *Gypsophila arrostii* Guss. var. *nebulosa* (Boiss. & Heldr.), *Gypsophila pilulifera* Boiss. & Heldr. and *Ankyropetalum reuteri Fenzl.* Boiss. on EG from *T. atroviride*. It was suggested that these natural saponins as non-ionic surfactants can be used together with cellulase in the industrial process. It is known that using cellulase in industry makes the process highly costly. The enzyme loading could be reduced by adding an appropriate concentration of these total saponins in the reaction mixture. In this way, the process could be economically viable.

Effect of the EG on the pretreated lignocellulosic waste

In this study, the influences of two various chemical pretreatments, as alkaline and acidic, have been compared. The effects of EG on sawdust and WB pretreated with NaOH and H_2SO_4 are shown as the amount of reducing sugars (mg g^{-1}) in Table 4. The results indicated that pretreatment with different concentrations of NaOH and H_2SO_4 significantly (p<0.05) altered the amount of reducing sugars released from poplar and WB compared to the untreated control (Table 5). While the EG had no effect on non-pretreatment sawdust, had little effect on the non-pretreatment WB. The released amount of reducing sugars was maximal for poplar and WB pretreated with alkali method. Previously, it has been stated that alkaline pretreatments are more effective than acidic pretreatment, and pretreatment

Table 4 – *Effects of EG to sawdusts and WB pretreated with NaOH and H_2SO_4*

	Poplar	Wheat bran	Calabrian pine	Abies pine
	Amount of reducing sugar (mg g^{-1})	Amount of reducing sugar (mg g^{-1})	Amount of reducing sugar (mg g^{-1})	Amount of reducing sugar (mg g^{-1})
control (distilled water)	0±0.00	4.6±0.40	0±0.00	0±0.00
0.5 M NaOH	6±0.50	15.4±1.47	0±0.00	0±0.00
1 M NaOH	6.4±1.13	16±0.23	0±0.00	0±0.00
0.05 M H_2SO_4	3±0.67	10.4±0.57	0±0.00	0±0.00
0.1 M H_2SO_4	3.4±0.35	12.4±0.44	4±0.07	3±0.26

Table 5 – *Statistical comparisons of the pretreatment methods*

Pretreatment method		*p* value			
		Poplar	Wheat bran	Calabrian pine	Abies pine
Control-alkali pretreatment	0–0.5 M NaOH	+	+	-	-
	0–1 M NaOH	+	+	-	-
Control- acid pretreatment	0–0.05 M H_2SO_4	+	+	-	-
	0–0.1 M H_2SO_4	+	+	+	+
Alkali-acid pretreatment	0.5 M NaOH–0.05 M H_2SO_4	+	+	+	+
	1 M NaOH–0.05 M H_2SO_4	+	+	+	+
	0.5 M NaOH–0.1 M H_2SO_4	+	+	+	+
	1 M NaOH–0.1 M H_2SO_4	+	+	+	+
Alkali-alkali pretreatment	0.5 M–1 M NaOH	-	-	-	-
Acid-acid pretreatment	0.5 M NaOH–0.05 M H_2SO_4	-	-	+	+

'+' The mean difference is significant, '-' the mean difference is not significant. The 95 % confidence level was set at $p < 0.05$.

with sodium hydroxide has attracted most attention.[52,53] All pretreatment methods used, except for 0.1 M H_2SO_4, had not significantly altered the amount of reducing sugars released from calabrian pine and abies pine compared to the non-treated control (p>0.05).

Poplar is hardwood (contains 40–55 % cellulose, 24–40 % hemicellulose, 18–25 % lignin), calabrian pine and abies pine are softwood (contains 45–50 % cellulose, 25–35 % hemicellulose, 25–35 % lignin).[44,50,51] It is suggested that, because poplar contains more cellulose and less lignin than the other, the removal of lignin using pretreatment methods can be easier and more effective than from other sawdust. From this point of view, WB contains less lignin than wood sawdust; therefore there has been an efficient increasing amount of released reducing sugars after pretreatment.

In conclusion, the concentration of H_2SO_4 had a significant (p<0.05) effect on hydrolysis of calabrian pine and abies pine. Increasing the concentration of H_2SO_4 from 0.05 M to 0.1 M significantly increased the released amount of reducing sugars. The effectiveness of acid and alkali pretreatment methods was not concentration-dependent for poplar and WB (Table 5).

The millions of tons of lignocellulosic wastes produced worldwide, especially WB and sawdust obtained from agricultural industries, is an important source for the production of renewable fuels.[1] Finally, we may say that the enzyme could benefit industrial processes such as the production of bioethanol used as economic and ecologic biofuel for transportation purposes.

Denim fabric treatment with the EG

In this study, the EG from *T. atroviride* was applied on a desized denim fabric and determined was its efficiency in producing the old look and the discolorization of the fabric (Fig. 10a and 10b). The cotton fabric treated with enzyme in Fig. 10b shows a more discolored surface than the fabric in Fig. 10a (no treatment with enzyme). Acidic cellulases are most commonly used in bio-stoning.[54] The EG from *T. atroviride* was acidic enzyme and caused discoloration of the denim fabric, thus producing the old look.

Fig. 11a and 11b shows the SEM images of the untreated denim fabric and denim fabric treated with EG, respectively. It seems that the desized denim fabric (Fig. 11a) contains huge amounts of fuzz and pill on the surface. After EG treatment, the fuzz and pill on the denim fabric was removed from the surface (Fig. 11b). The SEM images suggest that the EG had not significantly disrupted the inner fibers of the treated denim fabric (Fig. 11b) because the inner fibers are less accessible to EG.[55]

Cellulases provide a better finishing procedure by removing fuzz and pill on the denim surface. In addition, they have been used in softening, defibrillation, an in the process of providing localized differences in the color density of fibers.[54] Therefore, our results showed that the EG from *T. atroviride* had an effect for removing the fuzz indigo dye from the denim fabric and increased its softness. The obtained enzyme can be used for biostoning and denim finishing, without a notable harmful effect on the inner fibers. Similar results have been obtained by Montazer and Maryan.[55,56,57]

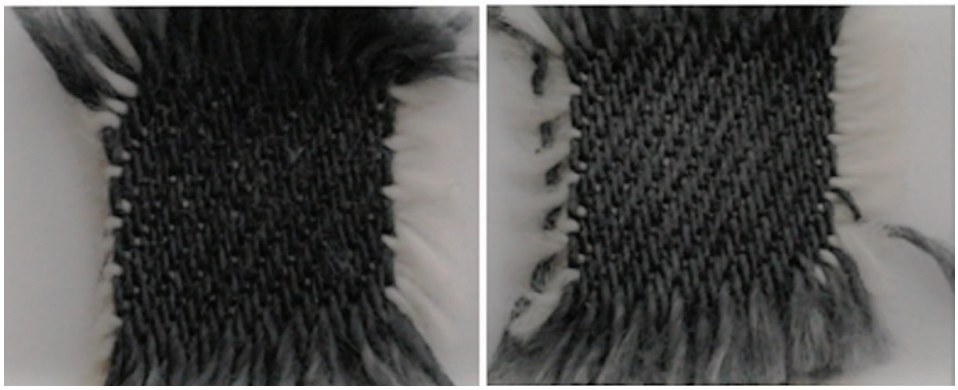
Conclusions

The EG was obtained and characterized from a local isolate *T. atroviride*. Performed were SDS PAGE, native PAGE, and zymogram test. The EG had high specific activity toward CMC compared to

Table 6 – *Comparison to EG obtained from previous studies with this EG*

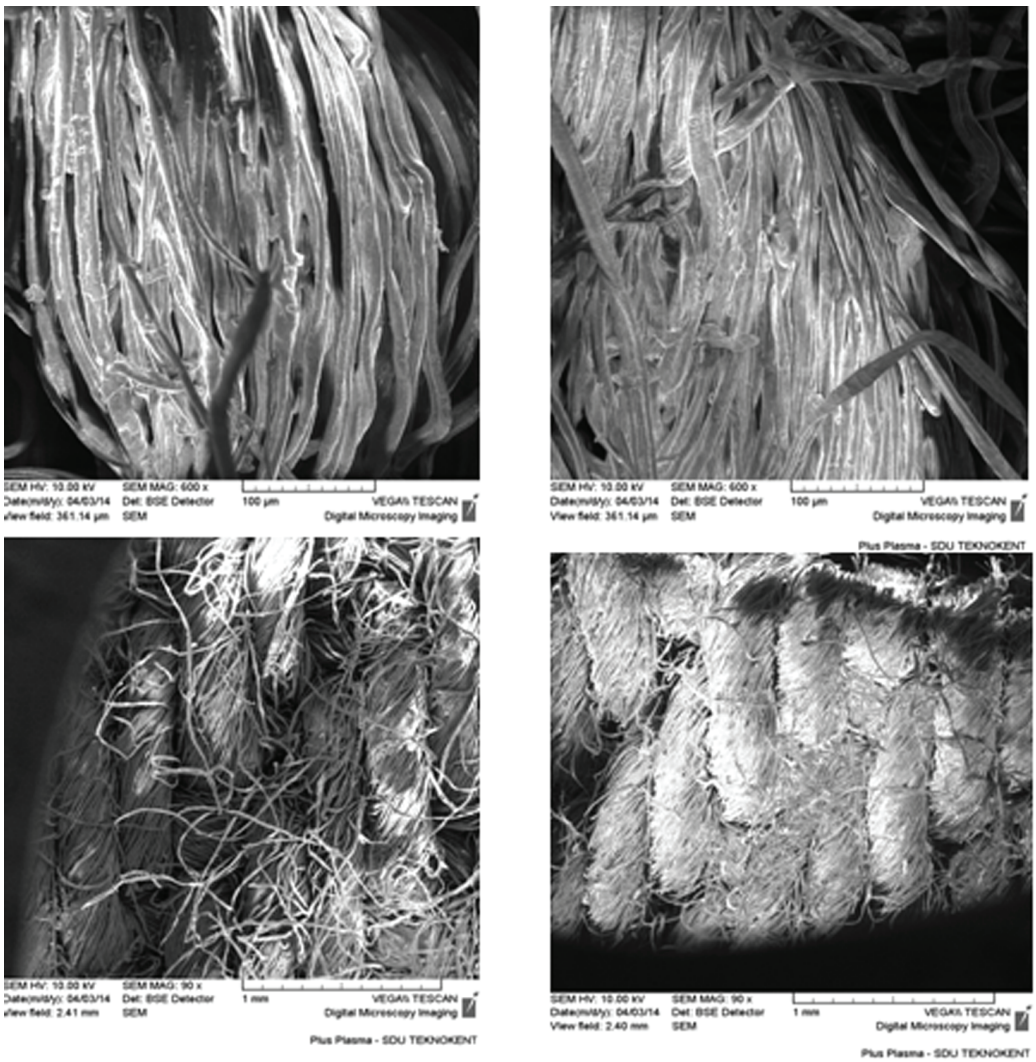
fungus	optimum pH	optimum temperature	thermal stability	K_m	ref.
C. sp. YJ5	7.0	60 °C	after 30 min incubation at 60 °C, retained activity 70 %	–	40
A. glaucus XC9	4.0	50 °C	after 1 h incubation at 60 °C, retained activity 60 %	5 mg mL^{-1}	5
A. aculeatus	5.0	40 °C	after 1 h incubation at 60 °C, retained activity 60 %	–	32
C. thermocellum	7.0	70 °C	after 48 h incubation at 60 °C, retained activity 50 %	–	60
T. harzianum ETS 323	4.5	50 °C	after 10 days incubation at 38 °C, retained activity 100 %	23 mg mL^{-1}	28
Penicillium pinophilum	5.0	50 °C	after 6 h incubation at 50 °C, retained activity 100 %	4.8 mg mL^{-1}	61
Mucor circinelloides	5.0	55 °C	after 30 min incubation at 70 °C, retained activity 90 %	–	62
T. atroviride 676	4.0	60 °C	after 8 h incubation at 50 °C, retained activity 90 %	–	20
T. atroviride	5.0	50 °C	after 72 h incubation at 50 °C, retained activity 90.5 %	1.12 mg mL^{-1}	in this study

-: not reported



a **b**

Fig. 10 – *Photos of old-look appearance and discolorization of the fabric. (a) Only treated with 0.05 M Na-citrate buffers (pH 5.0). (b) Treated with EG in 0.05 M Na-citrate buffers (pH 5.0).*



a **b**

Fig. 11 – *SEM images of denim fabric. (a) Only treated with 0.05 M Na-citrate buffers (pH 5.0). (b) Treated with EG in 0.05 M Na-citrate buffers (pH 5.0).*

EGs from *A. aculeatus*,[32] *A. glaucus* XC9,[5] *A. niger*,[58] *Pseudomonas fluorescens*,[59] *Clostridium thermocellum*,[60] *T. harzianum* ETS 323,[28] and optimum activity at pH 5.0 and 50 °C. The EG sustained its activity up to 72 h at 50 °C and pH 5.0, it lost only 9.5 % of its initial activity. The obtained EG had adequate properties such as low K_m and high thermal stability for industrial applications compared to EG obtained from previous studies (Table 6).

While $MgCl_2$ and $MgSO_4$ inhibited the enzyme, NaCl, Tween 80, Triton X-100 and total saponins from various plants activated it. Depending on the results obtained from biostoning and hydrolysis of pretreated lignocellulosic waste, it was suggested that the EG has useful properties for industrial applications and commercial purposes.

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