

# Production, Optimization, Partial Purification, and Characterization of a Novel Cellulase from *Bacillus subtilis* 171ES and Its Potential for Use in Textiles

Sarra Msakni, Elif Demirkan\* & Nilay Aladag Tanik

Bursa Uludag University, Faculty of Arts and Sciences, Department of Biology, Bursa, Turkey

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One hundred *Bacillus* sp. strains isolated from different soils were screened for cellulase production. A strain that produced maximum cellulase amounts was selected and identified by 16S rRNA analysis and was named *Bacillus subtilis* 171ES. In order to quantitatively determine the enzyme production capacity of the new isolate, it was grown in 3 different media. Optimization studies were carried out in the best medium. The results of nutritional optimization in the best liquid media showed that the best nitrogen and carbon sources, and metal ions were found to be milk powder, maltose and  $\text{LiSO}_4$ , respectively. In the study, enzyme production increased 6.6 times in the newly created modified medium. The enzyme was partially purified and characterized. The Optimum pH and temperature were 7 and  $50^\circ\text{C}$ , respectively. The  $\text{BaCl}_2$  and  $\text{MnSO}_4$  and ions were effective on activity. The kinetic values of the enzyme were  $V_{\max}$  63.7 U/mL and  $K_m$  0.0064 mM. The molecular weight was estimated as 63 kDa. The potential of the enzyme for biostoning on denim fabric and removal of the cellulosic or vegetative impurities on wool fabric was evaluated. It was also found to be effective in fabrics. The cellulase enzyme of the new 171ES strain has the potential to be used in various textile processes.

**Keywords:** Biostoning, Enzyme, Optimization, Thin layer chromatography, Vegetative impurities

## Introduction

The demand for industrial enzymes is constantly increasing due to the increasing need for sustainability solutions. Microorganisms are the most important sources for enzymes due the fact that their enzyme production is more stable and active than that of plant and animal enzymes. Additionally, they are amenable to large-scale cultivation with short fermentation period.<sup>1</sup> Microbial enzymes have wide-ranging uses in sectors such as food, textile, pharmaceutical, cosmetics, animal feed, waste treatment and agriculture. Their safety, reliability, stability, biodegradability, and ability to function under mild conditions make enzymes a preferred alternative to harsh chemicals in industrial processes.<sup>2</sup> Carbohydrases, including amylases, cellulases, glucanases, and others, rank among the top-selling enzymes globally, following proteases. Cellulase, a key carbohydrase, holds a significant market share of 32.84% vigour and finds diverse applications across industries such as detergent and laundry, textile, animal feed, biofuel, pulp and paper, food, medicine, and beverages.<sup>3</sup> According to a Market Research Report, the cellulase market is projected to experience a compound annual growth rate (CAGR) of

5.5% between 2018 and 2025, with anticipated revenues reaching USD 2.3 billion by the end of 2025.<sup>4</sup> Enzymes have emerged as a sustainable alternative to harsh and toxic chemicals commonly used in various sectors of the textile industry. In conventional textile manufacturing, known as wet processing, a series of steps involve the use of significant quantities of chemical agents like solubilizers, cleansing agents, defoamers, lubricants, surfactants, and dyes.<sup>5</sup> The discharge of these harmful chemicals into wastewater poses a significant environmental challenge. Consequently, the use of enzymes in textile industry is increasing day by day due to the harmful effects of chemicals. The main enzymes utilized in textile processing include pectinases, amylases, cellulases, lipases, proteases, catalases, and laccases. Among these, cellulase stands out as the most prevalent and versatile enzyme employed in wet textile processing for tasks such as biopreparation, softening cellulosic fibers, and biopolishing.<sup>6</sup>

Cellulases play a crucial role in various textile applications, including biostoning to remove excess dye from fabrics, enhancing colour brightness, softening cotton fibers.<sup>7</sup> On the other hand, it is used for biological cleaning in removing plant-derived substances consisting of thorns, grasses, leaves, and

\*Author for Correspondence  
E-mail: edemirkan@uludag.edu.tr

feed residues in wool, and also was used strength resistance.<sup>8</sup> The use of newly discovered cellulase enzymes as an alternative to chemicals in textile applications increases the importance of an environmentally friendly approach. There are studies in the literature on biostoning with cellulase obtained from different bacteria, but there are limited studies on cellulase from *Bacillus* spp. On the other hand, there is no research on *Bacillus* cellulase in the literature regarding the removal of plant-derived substances from wool. Therefore, the discovery of new efficient enzymes holds promise as a potential solution in textiles.

Cellulases break down  $\beta$ -1, 4-glucan bonds in cellulose and belong to the hydrolase enzyme group. They are naturally produced by microorganisms like fungi, bacteria, and protozoans, with *Bacillus* bacteria being a commonly utilized microorganism in biotechnology due to their ease of isolation and rapid growth capabilities, which can contribute to cost-effective cellulase production.<sup>9</sup>

Currently, novel microbial enzymes are being discovered from natural sources through various methods, including isolating efficient strains from existing microorganisms through mutation or utilizing existing microorganisms to obtain recombinant enzymes via recombinant DNA technology. Furthermore, optimizing production medium and fermentation conditions are important to increase enzyme production.

This study focused on evaluating the cellulase production potential of one hundred *Bacillus* strains isolated from soil and maintained in our Biotechnology Culture collection. The research involved optimizing the production environment, partially purifying and characterizing the enzyme, and investigating its efficacy in biostoning and removing plant residues in the wool industry.

## Materials and Methods

### Materials

One hundred *Bacillus* sp. strains used in this study were obtained from our previous studies.<sup>10</sup> The 100% wool fabrics contaminated with plant substances and denim fabric were provided from University of Bursa Uludag, Textile Engineering Department.

### Methods

#### Screening and Identification of Cellulase Producing Bacteria

*Bacillus* sp. strains were screened for cellulase activity on carboxymethyl cellulose (CMC) agar

plates containing (g/L) CMC 1, tryptone 10, yeast extract 5, NaCl 10, agar 15 (pH 7).<sup>11</sup> After 24 hours of incubation at 37°C, the surface of the petri dish was stained with 0.1% Congo red. Colonies that formed a clear hydrolysis zone in the Petri dish were identified as cellulase positive. The diameters of the colonies and hydrolysis zones were measured using a ruler, and the Enzymatic Index (EI) was calculated using the formula below.<sup>12</sup>

$$EI = \frac{\text{Diameter of hydrolysis zone}}{\text{Diameter of colony}} \quad \dots (1)$$

Microbial identification of strain 171ES genomic DNA was extracted from strain strain 171ES using the Fast DNA kit (Qbiogene, Montreal, PQ, Canada). The 16S rRNA gene was amplified from genomic DNA by polymerase chain reaction (PCR) using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTACCTTGTTACGACTT-3'). The amplified 1.449 bp sequences were determined by an automated ABI PRISM 3730 XL DNA analyzer (Applied Biosystems, Foster city, CA, USA). The resulting sequences were compared with the GenBank database (NCBI) using BLAST.<sup>13</sup> Sequences showing a relevant degree of similarity were imported into the CLUSTAL W program and aligned.<sup>14</sup> The evolutionary distances with other strains of *Bacillus* were computed using the neighbor-joining method and the phylogenetic relationships were determined using the software MEGA version (6.0).<sup>15</sup> The sequence of 171ES strain was submitted to the gene bank and the accession number was taken.

#### Production of Cellulase

The enzyme production medium consisted of the following components<sup>16</sup> (%w/v): CMC 1, MgSO<sub>4</sub> 0.03, yeast extract 0.6, K<sub>2</sub>HPO<sub>4</sub> 0.2, peptone 1, (NH<sub>4</sub>) SO<sub>4</sub> 0.25 (pH 7). Overnight culture with an optical density at 600 nm (OD<sub>600</sub>) of 0.3 were inoculated at 1% ratio into 150 mL of the medium in 500 mL Erlenmeyer flask and incubated for between 16 and 72 hours at 150 rpm and 37°C. After incubation period, the culture was centrifuged (3461 x g, 10 min) and the resulting crude enzyme-containing supernatant was collected for cellulase activity assays. Bacterial growth was monitored by measuring the optical density at 600 nm using a spectrophotometer (Beckman Coulter-UD 700).

#### Enzyme Assay

Dinitrosalicylic Acid (DNS) method was used for the determination of cellulase activity.<sup>17</sup> A reaction

mixture consisting of 0.5 mL of crude enzyme solution and 0.5 mL of substrate (1% CMC in 50 mM sodium phosphate buffer, pH 6.5) was incubated at 37°C for 30 min. The reaction was terminated by adding 1 mL of freshly prepared DNS reagent. Then, the reaction tubes were placed in boiling water for 5 minutes. After cooling, the absorbance was measured at 540 nm using a spectrophotometer. To quantify the cellulase activity, a glucose standard curve was generated by preparing glucose solution at different concentrations. One unit (U) of enzyme activity was described as the amount of enzyme that released 1  $\mu$ mol glucose per minute under experimental conditions.

#### **Optimization of Nutritional Factors for Cellulase Production**

To optimize the nutritional factors for cellulase production, the impact of various carbon sources including glycerol, sucrose, fructose, maltose, and starch, each at a concentration of 1% (w/v), was investigated as alternatives to the basal CMC substrate.

For organic nitrogen sources, skimmed milk powder, meat extract, peptone, yeast extract, tryptone were evaluated, while inorganic nitrogen sources such as KNO<sub>3</sub>, NH<sub>4</sub>Cl, NH<sub>4</sub>NO<sub>3</sub>, NaNO<sub>3</sub>, (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> were tested. These nitrogen sources were incorporated into the medium at the same levels as the existing organic (1.6% w/v) and inorganic (0.25% w/v) sources.

The effect of different metal ions including (0.03%) MnSO<sub>4</sub>, CaCl<sub>2</sub>, LiSO<sub>4</sub>, FeSO<sub>4</sub>, KCl and NaCl were also assessed for their influence on cellulase production.

Following experimental studies, a modified medium was formulated by combining the nutritional factors that yielded the maximum cellulase activities. The enzyme production of the bacteria cultivated in this optimized medium was compared to that of the basal medium in terms of yield.

#### **Partial Purification of Cellulase and Characterization**

Following bacterial cultivation, the supernatant obtained by centrifugation of the culture medium (+4°C, 10 min, 6000 rpm) was utilized as the source of the crude enzyme. The supernatant was subjected to precipitation through 60–90% ammonium sulfate fractionation. The resulting precipitate was collected by centrifugation (10,000 rpm) at +4°C for 30 min and dissolved in 50 mM sodium phosphate buffer (pH 6.5) buffer containing 1 mM CaCl<sub>2</sub>, and then dialyzed

overnight against the same buffer at +4°C. The dialyzed sample was concentrated by using ultrafiltration (Amicon, MW cut-off 30,000). The protein assay was performed by the Lowry method.<sup>18</sup>

To determine the optimal temperature for cellulase, the enzyme was incubated within the range of 35–80°C (pH 7). The optimal pH was determined by using various buffers; 0.1 M glycine–HCl (pH 2–3), 0.1 M sodium acetate (pH 4–6), 0.1 M Tris–HCl (pH 7–8), and 0.1 M glycine–NaOH (pH 9–10) at 37°C. The stability of the enzyme at different temperatures and pH levels were also assessed.

The impact of various salts, metal ions and reducing compounds on enzyme activity was investigated using 1 and 5 mM FeSO<sub>4</sub>, MnSO<sub>4</sub>, CaCl<sub>2</sub>, ZnSO<sub>4</sub>, MgSO<sub>4</sub>, CuSO<sub>4</sub>, BaCl<sub>2</sub>, LiSO<sub>4</sub>, KCl, NaCl, 2–Mercaptoethanol, EDTA and SDS. The relative activities (%) were calculated by taking the untreated enzyme activity as 100%.

For kinetic analysis was evaluated at 0.001–0.2 mM CMC concentrations. The K<sub>m</sub> and V<sub>max</sub> were calculated from the Lineweaver-Burk plot. The molecular weight of the enzyme was estimated by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS–PAGE).<sup>19</sup> The protein marker between 11–190 kDa was used as a standard to determine the molecular weight (Cell Signalling Technology, Prestained Protein Marker, #13953S). Additionally, the storage temperature stability of the crude cellulase was assessed by monitoring the enzyme activity after storage at +4°C, –20°C and room temperature (RT) for 120 days, with residual activities measured every 15 days.

#### **Determination of Carbohydrates by TLC Method**

The carbohydrates resulting for the hydrolysis of cellulose were separated and visualized using Thin Layer Chromatography (TLC)<sup>20</sup> on silica gel plates (20 cm x 20 cm size and 0.5 mm thick, Merck100390). A 1% CMC substrate solution was treated with 1 mL of crude enzyme and incubated for 10 h at 37°C. After incubation, 5  $\mu$ L of the reaction mixture was applied to the silica gel plate using a micro syringe (Hamilton). A solvent mixture of distilled water: ethanol: butanol (2:3:5) were used to develop the TLC plate. After solvent treatment, the plate was removed and dried at 110°C for 10 minutes and then sprayed with a solution of sulfuric acid and methanol (1:3, v/v). The plate was dried again at 110°C for 10 minutes, resulting in the visualization of hydrolysis products as black or brown spots on the

white plate. Glucose (G1) and cellobiose (G2) were used as standards for comparison.

#### *The Removal of the Cellulosic or Vegetative Impurities in Wool Fabrics*

Fabrics were treated with lyophilized (LAB312 brand TOPT-10 Model, 48 hours at  $-55^{\circ}\text{C}$ ) cellulase enzyme to remove vegetative impurities embedded in raw wool (100%). For this purpose, 5 cm diameter wool fabric pieces were placed in sterile petri dishes and treated with lyophilized cellulase for 1 hour at  $37^{\circ}\text{C}$  under both pH 4 and pH 7 conditions. After incubation, the fabric pieces were dried at room temperature. The removal of cellulosic or vegetative impurities from the wool fabrics was assessed using a light microscope (Nikon Eclipse E1000) for imaging and comparison with control samples. Additionally, manual rubbing of the fabrics after drying was performed to determine any further removal of impurities, with subsequent imaging and comparison with the control samples under the light microscope.

#### *Biostoning*

Biostoning was conducted as described previously.<sup>21</sup> The denim fabric was pre-washed with detergent at  $60^{\circ}\text{C}$  for 10 minutes, and then cut into  $5 \times 5$  cm pieces. These fabric pieces were divided into three groups and placed in separate beakers. The first beaker contained 75 mL of 50 mM sodium phosphate buffer (pH 7) and 25 mL of lyophilized raw cellulase, the second beaker contained water as a control, and the third beaker contained 10 mM NaOH. All beakers were incubated for 30 minutes at 100 rpm at  $50^{\circ}\text{C}$ . After the incubation period, the fabrics were transferred to 100 mL of 10 mM NaOH and left at room temperature for 10 minutes. Subsequently, the fabrics were rinsed with tap water for 2 minutes to remove NaOH dried at  $105^{\circ}\text{C}$  for 1 hour, and air-dried overnight at room temperature. Color changes were observed in both fabrics and beakers.

#### *Statistical Analysis*

For statistical analysis, the standard deviation for each experimental result and student's t-test was calculated using Excel Spreadsheets available in Microsoft Excel.

## **Results and Discussion**

#### **Cellulase Screening and Identification of Cellulase Positive Strain**

One hundred *Bacillus* strains were screened for their extracellular cellulase production capacity, with

54 strains identified as potential cellulase producers. Among these, 35 strains exhibited weak hydrolytic zones (EI = 1–1.5), 11 showed medium zones (EI = 1.6–1.9), and 8 displayed large zones (EI = 2–3). A strain with EI of 3 was selected and named 171ES (Fig. 1a, b). The sequencing of the 171ES strain gene for 16S rRNA bacterial identification was performed. Genomic DNA of 171ES strain was obtained using the Qiagen Blood & Tissue kit. Sequences were run with the ABI 3100 Genetic Analyzer device using universal primers and compared with other *Bacillus* species by looking at NCBI.<sup>13</sup> Alignment was done with ClustalW.<sup>14</sup> A phylogenetic tree was created using the neighbor-joining method with MEGA (6.0).<sup>15</sup> Subsequent sequencing of the 16S rRNA gene of strain 171ES confirmed its identification as *Bacillus subtilis*, with a 99% similarity to known sequences. This isolate was deposited in GenBank under the accession number OM004561 and designated as *B. subtilis*171ES (Fig. 1c).

#### **Enzyme Production**

A quantitative cellulase activity assay was performed. The maximal enzyme production and biomass concentration of 171ES strain were recorded as 68 U/mL and  $\text{OD}_{600} = 1.8$ , respectively, after 40 hours of incubation in the medium. Maximal enzyme

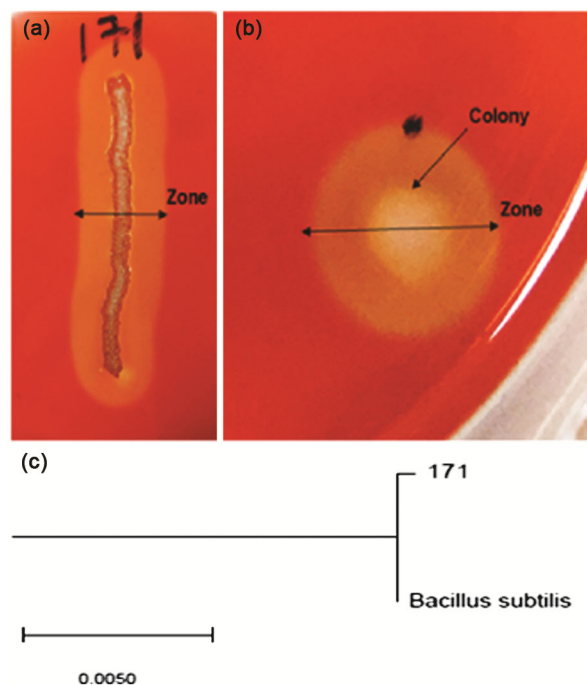


Fig. 1 — Qualitative determination of cellulase by streaking (a) and single colony (b) of *Bacillus* sp. 171ES on CMC agar plate, Phylogenetic tree of strain 171ES (c)

production was observed during the stationary phase. Previous studies have reported varying times for maximum cellulase production with different *Bacillus* species. Maximum cellulase production was achieved in 72 hours in *Bacillus* Y3<sup>(22)</sup>, while it was reported in 48 hours in *Bacillus cellulosilyticus*.<sup>16</sup>

#### Effect of Nutritional Factors on Cellulase Production

The synthesis of enzymes is influenced by the composition of the medium, particularly the presence of nitrogen and carbon sources. Therefore, optimizing the critical components of the medium is essential for enhancing the production of desired products. Additionally, metal ions play a crucial role in protein folding and the formation of the enzyme's active site.<sup>23</sup>

In this study different carbon sources were evaluated to determine the most effective one. The maximum enzyme production (428 U/mL) was achieved in the medium containing maltose, representing a 6.3-fold increase compared to the basal medium (68 U/mL, control). The preference order for carbon sources in enzyme production by strain 171ES was as follows; maltose > starch > sucrose > fructose > glycerol > control. Regarding bacterial growth, the preference order for carbon source was determined as maltose = starch = glycerol > control > fructose = sucrose (Fig. 2a).

Various organic and inorganic nitrogen sources were also tested to identify the optimal nitrogen source. The organic and inorganic nitrogen sources preference of 171ES were Skim Milk > Yeast Extract > Peptone = Meat Extract > Tryptone > Control >  $(\text{NH}_4)_2\text{HPO}_4 = \text{NH}_4\text{NO}_3 = \text{NaNO}_3 > \text{NH}_4\text{Cl} > \text{KNO}_3$ . When yeast extract and peptone were used separately, the enzyme yield increased by 73% and 47%, respectively, compared to the basal medium. The highest enzyme production (532 U/mL) was observed in the presence of skim milk powder representing a 7.8-fold increase compared to the control. Organic nitrogen sources were found to be more suitable for cellulase production. In terms of bacterial growth, the preferred order for nitrogen sources was Meat Extract > Yeast Extract > Control (basal medium) = Tryptone > Peptone = Skim Milk Powder >  $\text{KNO}_3 > \text{NH}_4\text{Cl} = \text{NH}_4\text{NO}_3 = \text{NaNO}_3 > (\text{NH}_4)_2\text{HPO}_4$  (Fig. 2b).

Furthermore, metal ions were investigated for their stimulatory effect on cellulase production. Substituting  $\text{MgSO}_4$  (0.03%) in the medium with various metal ions revealed that cellulase enzyme production increased by 1.85 times (126 U/mL) in the presence of  $\text{LiSO}_4$  compared to the basal medium (68 U/mL). The preference order for metal ions in the enzyme production by the bacteria was determined as

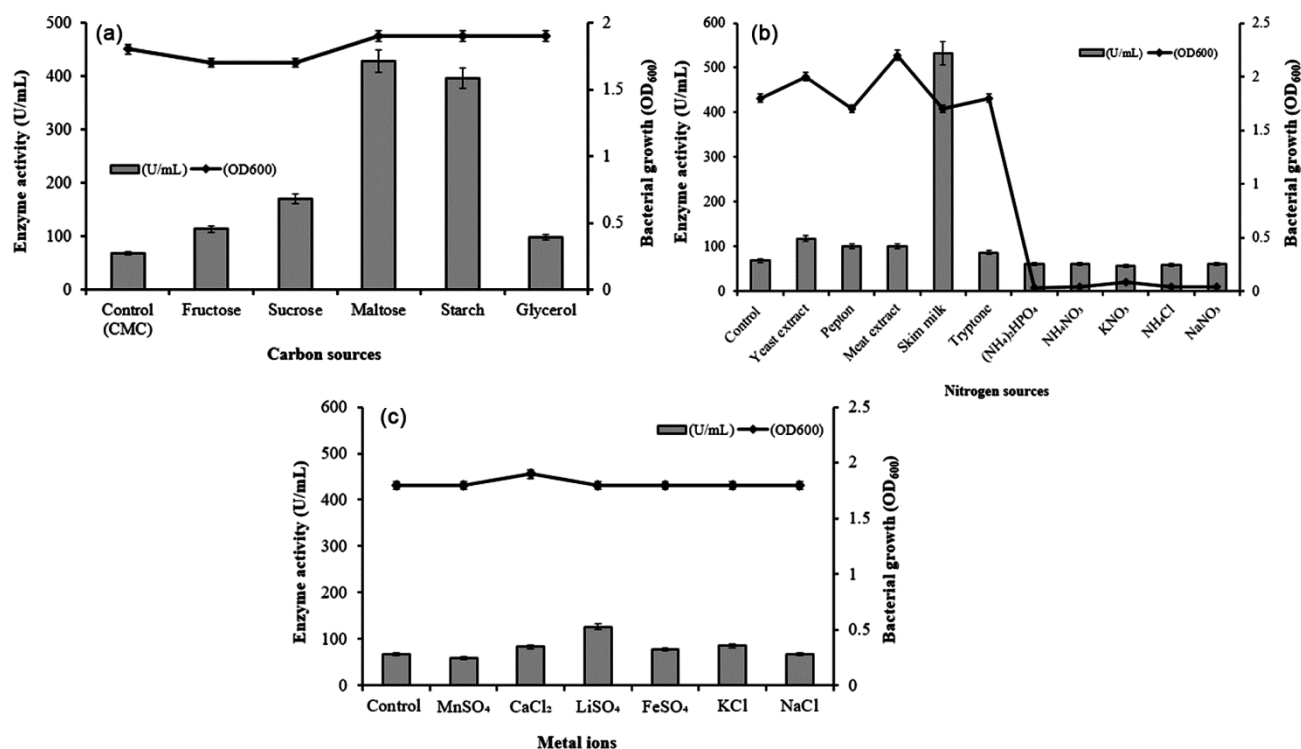


Fig. 2 — Effects of some carbon (a), nitrogen (b) and metal ions (c) sources on cellulase production and bacterial growth

$\text{LiSO}_4 > \text{KCl} > \text{CaCl}_2 > \text{FeSO}_4 > \text{NaCl} > \text{MnSO}_4$ , respectively. Whereas, the maximum bacterial growth was observed in the presence of  $\text{CaCl}_2 > \text{LiSO}_4 = \text{Control} = \text{KCl} = \text{NaCl} = \text{MnSO}_4 = \text{FeSO}_4$  medium (Fig. 2c).

A novel modified medium was formulated by combining the optimal nutritional components to enhance the overall cellulase enzyme yield. The modified medium comprised (% w/v): maltose 1, skim milk powder 1.6,  $\text{LiSO}_4$  0.03,  $\text{K}_2\text{HPO}_4$  0.2 (pH 7). In this modified medium, the enzyme yield increased by 6.6 times (453 U/mL) compared to the control (68 U/mL).

Several researchers have utilized various nitrogen sources, carbon sources, and metal ions in cellulase production. For instance, the maximum enzyme production by *Bacillus pumilus* EWBCM1 was achieved at 72 hours at 37°C in the presence of galactose (0.5851 IU/mL) and malt extract (0.5666 IU/mL). Among the metal ions tested, calcium chloride resulted in the highest enzyme production (0.1851 IU/mL).<sup>24</sup> In another study, cellulase production from *Bacillus subtilis* isolated from cow dung reached 23.96 U/mL with lactose and 29.63 U/mL with peptone.<sup>21</sup> Additionally, 2% CMC as a carbon source led to the maximum production of cellulase enzyme (34 U/mL) in *Bacillus subtilis*.<sup>25</sup> Maximum cellulase activity was found as 4.72 IU/mL for *Bacillus* sp. Y3 with CMC (4.49 IU/mL) and 1% peptone as the nitrogen source.<sup>22</sup> Additionally,

cellulase production from *Bacillus amyloliquefaciens* FW22 was enhanced by lactose, yeast extract and  $\text{Mg}^{+2(26)}$

The differences observed in cellulase production among various studies may be attributed to the distinct characteristics of the microorganisms, production conditions, and nutrient media utilized. Variation in metabolic pathways of microorganisms for enzyme production and experimental conditions can also contribute to differences in cellulase activity measurements.

#### Partial Purification and Characterization

The enzyme's activity is influenced by specific characteristic such as temperature, pH, metal ions,  $K_m$ , and  $V_{max}$ , which are crucial for its industrial applicability. Temperature and pH are key parameters affecting enzyme activity, while metal ions can either enhance or inhibit enzymatic activity by interacting with the carboxylic acid or amino group of amino acids.<sup>27</sup> The  $K_m$  and  $V_{max}$  values reflect the enzyme's kinetic properties, indicating its substrate affinity and maximum reaction rate. Enzymes with low  $K_m$  values are particularly valuable in industrial applications due to their high substrate affinity.

In this study, the enzyme was partially purified through precipitation with 80% ammonium sulfate, followed by dialysis and ultrafiltration, resulting a final specific activity of 500 U/mL with 4.8 purification fold. The yield obtained was 0.6%.

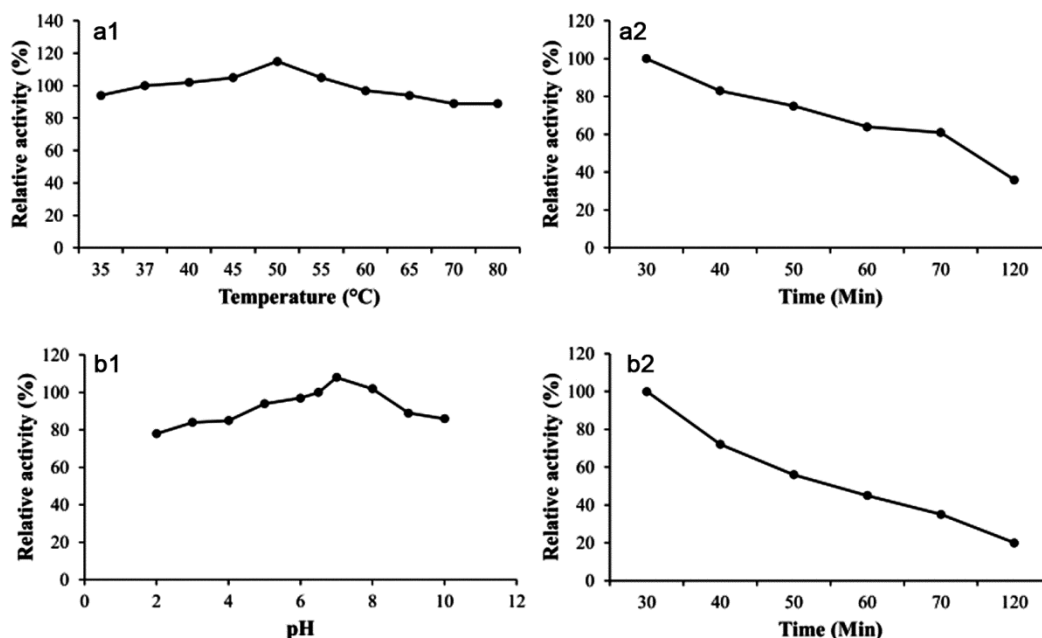


Fig. 3 — Effect of temperature on activity (a1) and stability (a2), pH value effect on activity (b1) and stability (b2)

The optimal temperature for the cellulase derived from *B. subtilis* 171ES was determined to be 50°C (Fig. 3a1), with thermobility studies showing that the enzyme retained 75% activity for 50 min at this temperature (Fig. 3a2). The enzyme exhibited an optimal pH of 7 (Fig. 3b1), and stability studies revealed that it retained 56% activity for 50 minutes at pH 7 (Fig. 3b2).

Various reducing compounds and metal ions were tested for their effect on enzyme activity, with most compounds at 5 mM concentration showing an increase in enzyme activity. Notably, BaCl<sub>2</sub> and MnSO<sub>4</sub> were found to have effective activator effects, while FeSO<sub>4</sub>, CuSO<sub>4</sub>, CaCl<sub>2</sub>, and KCl also positively influenced the enzyme. On the other hand, other metal ions had an inhibitory effect. Additionally, SDS, an anionic detergent, was found to effectively activate the enzyme. But other metal ions had an inhibitory effect. Metals have a positive/negative effect on the activity by binding either to the active center of the enzyme or to the structure outside the active center. The crystal structure of the *Bacillus* cellulase catalytic core has shown that two glutamate residues are located close to each other in the active site of the enzyme. Additionally, it has been reported that there is a serine–histidine–glutamate catalytic triad in the active center, which may be a new combination in the catalytic event.<sup>28,29</sup> Mn<sup>2+</sup> and Ba<sup>2+</sup> can reversibly bind to electronegative glutamate residues or this catalytic triad, causing positive changes in enzymatic catalytic activity. This may facilitate the hydrolysis of the glycosidic bonds of the substrate bound to the active site. On the other hand, SDS, an anionic surfactant, was also found to activate the enzyme effectively. SDS is thought to activate conformational transitions

of cellulase. Thus, it may have the ability to activate the enzyme by changing the enzymatic and physical properties of the enzyme. However, 2–Mercaptoethanol and EDTA inhibited the enzyme activity.

In this study, the molecular weight of cellulase produced by 171ES was evaluated as 63 kDa (Fig. 4a). When the kinetic properties of the enzyme were investigated, the V<sub>max</sub> value was calculated as 63.69 U/mL. K<sub>m</sub> value was found as 0.0064 mM (Fig. 4b).

Studies on cellulases from various *Bacillus* sp. have shown diverse properties. For instance, *Bacillus subtilis* YJ1 cellulase enzyme exhibited optimal activity at pH 6 and 50–60°C, with a molecular weight of 32.5 kDa. The enzyme's activity was enhanced by Co<sup>2+</sup> and Mn<sup>2+</sup> but inhibited by DTT, p–CMB, SDS, Fe<sup>2+</sup> and Fe<sup>3+</sup>, Cd<sup>2+</sup> and Hg<sup>2+</sup>.<sup>(30)</sup> Another study on *Bacillus subtilis* reported a 51.4 kDa enzyme with maximal activity at pH 8 and 45°C, showing stimulation by Ca<sup>2+</sup> and Na<sup>+</sup> metal ions. Surfactants like Tween 20, SDS and Triton X-100, have been observed to enhance the enzymatic activity of cellulase. K<sub>m</sub> and V<sub>max</sub> values of cellulase enzyme were reported as 1.97 mg/mL and 75.41 mg/mL/s, respectively.<sup>25</sup> On the other hand, the cellulase from *Bacillus vallismortis* RG-07 exhibited an optimal temperature of 65°C and pH of 7. The molecular weight was estimated as 80 kDa. Enzyme activity was induced by Tween–60, Ca<sup>2+</sup>, sodium hypochlorite and mercaptoethanol, while it was strongly inhibited by Hg<sup>2+</sup>. Kinetic analyses were reported that K<sub>m</sub> and V<sub>max</sub> were 1.923 mg/mL and 769.230 μg/mL, respectively.<sup>31</sup> Additionally, Afzal *et al.* (2019) revealed the characteristic features of *Bacillus licheniformis* HI-08 cellulase.<sup>32</sup> They determined that

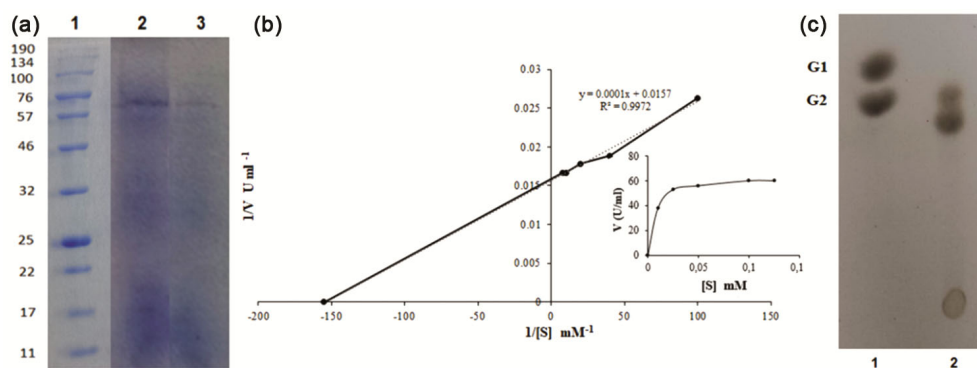


Fig. 4 — (a) Gel electrophoresis of cellulase; Lane 1. Protein marker (11–190 kDa, Cell Signalling Technology, Prestained Protein Marker, #13953S), Lane 2. Crude enzyme, Lane 3. Partially purified enzyme (b) Lineweaver–Burk plot and Michaelis–Menten graphs (c) TLC analysis of hydrolysis products of CMC substrate; Line 1: a mixture of glucose–G1 and cellobiose–G2, Line 2: products of CMC hydrolysis

the molecular weight of the enzyme was 55 kDa. The optimal temperature and pH were determined at 45°C and pH 7, respectively. Kinetic analysis of the purified HI-08 cellulase found  $K_m$  and  $V_{max}$  to be 2.28 mg/mL and 454.05  $\mu$ g/mL respectively. This cellulase enzyme exhibited increased activity in the presence of alkali metal ions such as  $Mg^{2+}$ ,  $Ca^{2+}$ , and  $Na^+$ , while being inhibited by  $Hg^{2+}$ ,  $Zn^{2+}$ ,  $Ni^{2+}$ ,  $Co^{2+}$ , and  $Cu^{2+}$ . These findings highlight the diverse properties of cellulases produced by different *Bacillus* species.

### Products of Hydrolysis of Cellulose by 171ES Cellulase

Through TLC analysis depicted in Fig. 4c, it was observed that the crude enzyme effectively degraded the substrate into cellobiose (G2) and presumably cellotriose (G3) as the final main product. When the short-spined sea urchin cellulase was incubated with various cellulosic substrates, and degradation products were detected by TLC. Cellotetraose, cellotriose and cellobiose were determined as the main products by the degradation of the phosphoric acid-swollen cellulose by sea urchin cellulase. It was noted that similar main products were generated from the hydrolysis of crystalline cellulose, although the enzyme displayed lower degradation activity on this substrate.<sup>33</sup>

### Storage Stability of the Crude Cellulase

In this study, the storage stability of the crude enzyme was assessed by monitoring its activities at room temperature (RT), +4°C, and -20°C every 15 days. The enzyme activity remained stable for 30 days at RT, and 75 days at +4°C, and up to 90 days at -20°C. Even after 120 days -20°C, there was only a 14% decrease in activity. It was found that the enzyme retained around 75% of its activity after 30 days of storage at 4°C, and approximately 40% at 25°C. Additionally, after 28 days of storage at RT, only 68% (1.10 U/mL) of the control activity (1.60 U/mL, 100%) was preserved.<sup>34</sup>

### Potential of Cellulase Enzyme in the Textile Industry

Raw wool fabrics contain high levels of plant residues as well as some impurities, which are traditionally removed through a process known as “wool carbonization” in the textile industry. This method involves converting cellulose into a more easily removable form called hydro cellulose using strong sulfuric acid. However, to avoid the use of harsh chemicals and reduce wool fiber damage, waste

generation, and energy consumption, enzymes are now being utilized.<sup>35</sup>

Jeans, whether dyed with natural or synthetic indigo, are a popular choice among individuals of all ages, with approximately a billion pairs produced annually. The traditional method of washing jeans with a pumice stone, known as stone washing, results in a faded appearance. However, the use of pumice stones can lead to issues such as stones crumbling, damage to machine parts and clogging of drainage system. In addition, excessive rubbing of the pumice stones on the fabric can cause abrasions. For this reason, enzymes have started to be used as an alternative for stone washing. It has been estimated that 80% of denim garments have been processed with cellulase enzymes for the last 20 years.<sup>36</sup> The enzymatic process is known as biostoning process. This enzymatic approach aims to achieve a more authentically aged appearance for denim garments while also offering environmentally friendly solution that eliminates the drawbacks associated with traditional pumice stone washing method.

In this study, the effects of cellulase enzyme of new isolate *Bacillus subtilis* 171ES on the removal of cellulosic or vegetable impurities in woolen fabrics and its biostoning effects on denim fabric were investigated. The efficacy of lyophilized cellulase in removing vegetable impurities from 100% wool fabrics was assessed using light microscopy. Initially, the soiled fabrics (Controls, Figs. 5a1 and b1) were examined under the microscope (controls) before being treated with the enzyme, did not completely remove the plant residues after 1 hour of incubation with the enzyme at different pH (Figs. 5a2 and b2). However, the size of the impurities decreased significantly after the manual rubbing of the fabrics (Figs. 5a3 and b3). The findings suggest that the cellulase enzyme weakens vegetable impurities during the one-hour incubation by breaking them down, but mechanical rubbing is necessary to fully eliminate the weakened residues. Extending the incubation period may further enhance the removal process. While industrial carbonization processes typically operate at pH 4 for plant waste removal, this study demonstrated improved outcomes at pH 7.

The enzyme application method and the conventional dilute sulfuric acid carbonization process were compared in another study and it was found that treatment of untreated wool fabric with sulfuric acid effectively eliminated vegetable matter

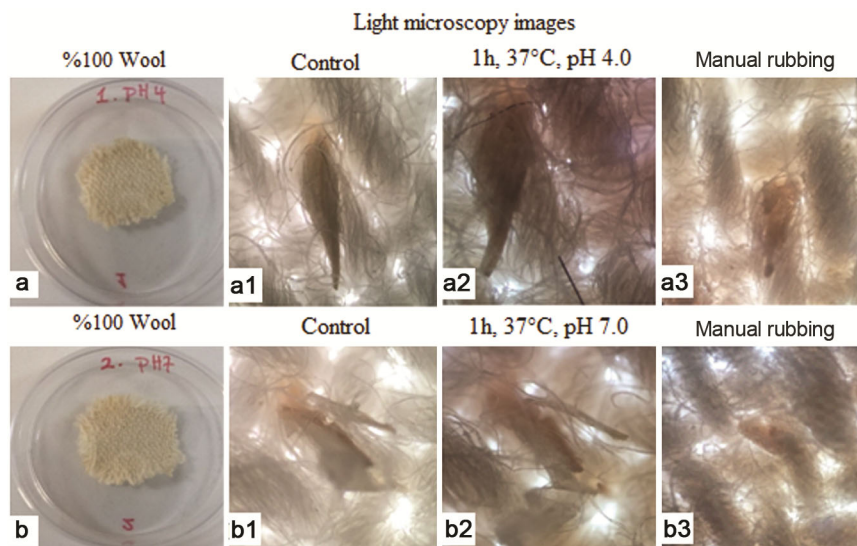


Fig. 5 — Light microscope images of the removal with cellulase of cellulosic or vegetable impurities from 100% wool fabrics; a–b: 100% wool fabric containing cellulosic or vegetable impurities, a1–b1: without applying enzyme (Control), a2–b2: incubation with enzyme, a3–b3: manual rubbing

from wool, but led to a decrease in tensile strength of carbonized fabric compared to untreated wool fabrics. On the other hand, treating wool with cellulases (Biotouch L) at a concentration of 20 mL/L removed approximately 75% of vegetable impurities and resulted in increased tensile strength compared to untreated and carbonized wool fabrics.<sup>8</sup>

When investigating the potential of cellulase from isolate 171ES as a biostoning agent, it was observed that incubating the enzyme at 50°C with denim fabric for 30 minutes resulted in bleaching of the fabric color. It was determined that cellulase enzyme effectively removed the indigo dye from denim fabric (Fig. 6). It was suggested that *Bacillus cereus* MRK1 cellulase enzyme was found to be efficient in removing color from denim fabric.<sup>37</sup>

## Conclusions

The study focused on isolating bacteria from nature that produce highly efficient enzymes, particularly cellulase. The cellulase production capacity of the bacteria that we previously isolated from the soil was investigated, with a specific emphasis on optimizing the nutritional production conditions of cellulase enzyme derived from the new isolate *Bacillus subtilis* 171ES. The enzyme was subjected to partial purification for characterization purposes.

Rather than relying on chemical processes commonly used in the textile sector, the study showcased the potential of utilizing this enzyme in a more environmentally friendly approach for biostoning

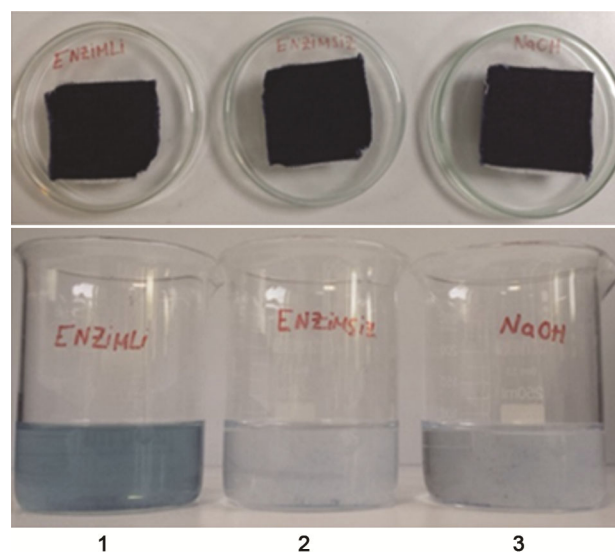


Fig. 6 — Biostoning of denim fabric with cellulase enzyme; 1–Enzyme treatment, 2–without enzyme treatment, 3–NaOH treatment

processes and the removal of the cellulosic or vegetative impurities from woolen fabrics. The cellulase enzyme derived from this organism demonstrated promising potential for applications in the textile industry and may also hold promise for various other industrial sectors.

## References

- 1 Anbu P, Gopinath S, Cihan A C & Chaulagain B P, Microbial enzymes and their applications in industries and medicine, *Biomed Res Int*, (2013) 204014, <https://doi.org/10.1155/2017/2195808>.

- 2 Kumar D, Bhardwaj R, Jassal S, Goyal T, Khullar A & Gupta N, Application of enzymes for an eco-friendly approach to textile processing, *Environ Sci Pollut Res*, **30(28)** (2021) 71838–71848, <https://doi.org/10.1007/s11356-021-16764-4>.
- 3 Li X, Chang S H & Liu R, Industrial applications of cellulases and hemicellulases, in *Fungal Cellulolytic Enzymes: Microbial Production and Application*, edited by X Fang & YQu (Springer, Singapore) 2018, 267–282.
- 4 Jayasekara S & Ratnayake R, Microbial cellulases: An overview and application, in *Cellulose*, edited by A R Pascual & M E E Martin (Intechopen, London) 2019, 1–21, <https://doi.org/10.5772/intechopen.84531>.
- 5 Gulzar T, Farooq T, Kiran S, Ahmad I & Hameed A, Green chemistry in the wet processing of textiles, in *Impact prospect. green chem. text*, edited by Shahid-ul-Islam & B S Butola (Woodhead Publishing, Sawston) 2019, 1–20, <https://doi.org/10.1016/B978-0-08-102491-1.00001>.
- 6 Hebeish A, Kamel M M, Helmy H M & El Hawary N S, Science-based options for application of cellulose biotreatment and reactive dyeing to cotton fabrics, *Life Sci*, **10(4)** (2013) 3281–3289.
- 7 Aygan A, *Haloalkalophile Bacillus sp. Isolation, Production and Characterization of Amylase, Cellulase and Xylanase Enzymes and their Usability in Biotechnological Applications*. Ph D Thesis, Cukurova University, Institute of Science and Technology, Adana, Turkey, 2008.
- 8 El-Sayed H, El-Gabry L & Kantouch F, Effect of biocarbonization of coarse wool on its dyeability, *Indian J Fibre Text Res*, **35(4)** (2010) 330–336.
- 9 Arusha P N, Kiran R K, Shanti G G & Arun S K, Optimization of cellulase production for *Bacillus* sp. and *Pseudomonas* sp. soil isolates, *African J Microbiol Res*, **10(13)** (2016) 410–419, <https://doi.org/10.5897/AJMR2016.7954>.
- 10 Demirkan E, Kut D, Sevgi T, Dogan M & Baygin E, Investigation of effects of protease enzyme produced by *Bacillus subtilis* 168 E6-5 and commercial enzyme on physical properties of woolen fabric, *J Text Inst*, **111(1)** (2020) 26–35, <https://doi.org/10.1080/00405000.2019.1624069>.
- 11 Lennete E H, Ballows A, Hausler J W Jr & Shadomy J H, *Manuel of Clinical Microbiology*, 4<sup>th</sup> edn, vol 4 (American Association for Microbiology, Washington DC) 1985, 11–49.
- 12 Florencio C, Couri S & Farinas C S, Correlation between agar plate screening and solid-state fermentation for the prediction of cellulase production by *Trichoderma* strains, *Enzyme Res*, (2012) 1–7, <https://doi.org/10.1155/2012/793708>.
- 13 Altschul S F, Gish W, Miller W, Myers E W & Lipman D J, Basic local alignment search tool, *J Mol Biol*, **215(3)** (1990) 403–10, <https://doi.org/10.1016/0022-2836/90/190403-08> \$03.00/0.
- 14 Thompson J D, Higgins D G & Gibson T J, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, *Nucleic Acids Res*, **22** (1994) 4673–4680, <https://doi.org/10.1093/nar/22.22.4673>.
- 15 Saitou N & Nei M, The neighbor-joining method: A new method for reconstructing phylogenetic trees, *Mol Biol Evol*, **4** (1987) 406–25, <https://doi.org/10.1093/oxfordjournals.molbev.a040454>.
- 16 Maryam B, Qadir A, Zameer M, Ahmad S R, Nelofer R, Jamil N, Arzoo S & Afzaal R, Production of cellulases by *Bacillus cellulosilyticus* using lignocellulosic material, *Pol J Environ Stud*, **27(6)** (2018) 2659–2667, <https://doi.org/10.15244/pjoes/80867>.
- 17 Miller G, Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal Chem*, **31** (1959) 426–428, <https://doi.org/10.1021/ac60147a030>.
- 18 Lowry O H, Rosebrough N J, Farr A L & Randall R J, Protein measurement with the folin phenol reagent, *J Biol Chem*, **193(1)** (1951) 265–275.
- 19 Laemmli U K, Paulson J R & Hitchins V, Maturation of the head of bacteriophage T4. V. a possible DNA packaging mechanism: *In vitro* cleavage of the head proteins and the structure of the core of the polyhead, *J Supramol Struct*, **2(2–4)** (1974) 276–301, <https://doi.org/10.1002/jss.400020219>.
- 20 Stahl E, *Thin-Layer Chromatography: A Laboratory Handbook* (Springer Science & Business Media, Berlin) 2013, 10–41.
- 21 Bai S, Kumar M R, Kumar D M, Balashanmugam P, Kumaran M D B & Kalaichelvan P T, Cellulase production by *Bacillus subtilis* isolated from cow dung, *Arch Appl Sci Res*, **4(1)** (2012) 269–279.
- 22 Lugani Y, Singla R & Sooch B S, Optimization of cellulase production from newly isolated *Bacillus* sp. Y3, *J Bioprocess Biotech*, **5(11)** (2015) 264, <https://doi.org/10.4172/2155-9821.1000264>.
- 23 Smith A M, Interaction of metal ions with proteins as a source of inspiration for biomimetic materials, in *Functional Metallo-supramolecular Materials*, edited by J G Hardy & F H Schacher (Royal Society of Chemistry, Cambridge) 2015, 1–31, <https://doi.org/10.1039/9781782622673-00001>.
- 24 Shankar T & Isaiarasu L, Cellulase production by *Bacillus pumilus* EWBCM1 under varying cultural conditions, *Middle East J Sci Res*, **8(1)** (2011) 40–45.
- 25 Asha B M & Sakthivel N, Production, purification and characterization of a new cellulase from *Bacillus subtilis* that exhibit halophilic, alkalophilic and solvent-tolerant properties, *Ann Microbiol*, **64(4)** (2014) 1839–1848, <https://doi.org/10.1007/s13213-014-0835-x>.
- 26 Pham V H T, Kim J, Shim J, Chang S & Chung W, Coconut mesocarp-based lignocellulosic waste as a substrate for cellulase production from high promising multienzyme-producing *Bacillus amyloliquefaciens* FW2 without pretreatments, *Microorganisms*, **10(2)** (2022) 327, <https://doi.org/10.3390/microorganisms-10020327>.
- 27 Ishida N, Okubo A, Kawai H, Yamazaki S & Toda S, Interaction of amino acids with transition metal ions in solution (I) solution structure of L-lysine with Co(II) and Cu(II) ions as studied by nuclear magnetic resonance spectroscopy, *Agric Biol Chem*, **44(2)** (1980) 263–270, <https://doi.org/10.1080/00021369.1980.10863934>.
- 28 Shaw A, Bott R, Vonnrhein C, Bricogne G, Power S & Day A G, A novel combination of two classic catalytic schemes, *J Mol Biol*, **320(2)** (2022) 303–309, [https://doi.org/10.1016/S0022-2836\(02\)00387-X](https://doi.org/10.1016/S0022-2836(02)00387-X).
- 29 Tambalo R D, Raymundo A K & Grunden A M, Thermostable endoglucanase gene derived by amplification from the genomic DNA of a cellulose-enriched mixed culture from mud spring water of Mt. Makiling, Laguna, Philippines, *World J Microbiol Biotechnol*, **36(3)** (2020) 1–4, <https://doi.org/10.1007/s11274-020-02825-2>.
- 30 Yin L J, Lin H H & Xia Z R, Purification and characterization of a cellulase from *Bacillus subtilis* YJ1, *J Mar Sci Technol*, **18(3)** (2010) 466–471, <https://doi.org/10.51400/2709-6998.1895>.

- 31 Gaur R & Tiwari S, Isolation, production, purification and characterization of an organic-solvent-thermostable alkalophilic cellulase from *Bacillus vallismortis* RG-07, *BMC Biotechnol*, **15(19)** (2015), [https://doi.org/ 10.1186/s12896-015-0129-9](https://doi.org/10.1186/s12896-015-0129-9).
- 32 Afzal M, Qureshi M Z, Khan S, Khan M I, Ikram H, Ashraf A & Qureshi N A, Production, purification and optimization of cellulase by *Bacillus licheniformis* HI-08 isolated from the hindgut of wood-feeding termite, *Int J Agric Biol*, **21(1)** (2019) 125–134, <https://doi.org/10.17957/IJAB/15.0872>.
- 33 Hasegawa S, Ura K, Tanaka H, Ojima T & Takagi Y, Purification and biochemical characterization of a cellulase from the digestive organs of the short-spined sea urchin *Strongylocentrotus intermedius*, *Fish Sci*, **78(5)** (2012) 1107–1115, <https://doi.org/10.1007/s12562-012-0528-y>.
- 34 Islam M, Sarkar P K, Mohiuddin A K M & Suzauddul M, Optimization of fermentation condition for cellulase enzyme production from *Bacillus* sp., *Malays J Halal Res*, **2(2)** (2019) 19–24, <https://doi.org/10.2478/mjhr-2019-0009>.
- 35 Gouveia I C, Fiadeiro J M & Queiroz J A, Combined bio-carbonization and dyeing of wool: a possibility using cell wall-degrading enzymes and 1:1 metal–complex dyes, *Eng Life Sci*, **8** (2008) 250–259, <https://doi.org/10.1002/elsc.200700055>.
- 36 Agrawal B J, Bio-stoning of denim-an environmental-friendly approach, *Curr Trends Biomedical Eng Biosci*, **3(3)** (2017) 555612, <https://doi.org/10.19080/CTBEB.2017.03.555612>.
- 37 Kumar D M, Poovai P D, Kumar C P, Saroja Y S, Manimaran A & Kalaichelvan P T, Optimization of *Bacillus cereus* MRK1 cellulase production and its Biostoning activity, *Sch Res J*, **4(3)** (2012) 881–888.