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RESEARCH ARTICLE

Isolation of a cellulase producing *Bacillus cereus* from cow dung and determination of the kinetic properties of the crude enzyme

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Abstract: The objective of the study was to isolate a thermostable alkaline cellulase producing bacterial strain from diverse natural sources such as goat excreta, cow dung, tropical soil, and organic matter. Opened hot cellulose agar plates were transferred on to the selective carboxy methyl cellulose (CMC) agar media and incubated for 2 days at 37 ^{o}C. The samples were cultured on Nutrient Agar in order to isolate cellulolytic bacteria. The bacteria isolated were screened for cellulolytic activity using serial dilution and pour-plate method after which they were characterised. The bacterial isolate with the highest CMC hydrolytic capacity which was obtained from cow dung was selected for further studies. Based on the morphological, biochemical and cultural analysis, the strain from cow dung was been identified as *Bacillus sp*. Pure culture of this bacterial strain was grown overnight, DNA was extracted and 16S rDNA was amplified by thermocycler using universal primers. When the amplified 16S rDNA PCR product was sequenced using automated sequencer and sequence similarity search was done for the 16S rDNA sequence using BLAST, the unknown organism was identified as *Bacillus cereus* with the GenBank accession no AF290555. Cellulase from *Bacillus cereus* showed zero order kinetics for 20 minutes. The optimum pH for the crude cellulase from *Bacillus cereus* was 9.0 at 45^{o}C and the temperature optimum was 45^{o}C at pH 9.0. Michaelis constant for the cellulase from this isolate to soluble cellulose was 38.60 $g^{-1}L$ and V_{max} was 3.32 µmol mL^{-1} min^{-1} at pH 9.0 and at 45 ^{o}C. Since this cellulase enzyme was stable for more than an hour at 45^{o}C and at alkaline pH values, it could be a good candidate for industrial applications.

Keywords: Alkaline cellulase, *Bacillus cereus*, CMC agar media, cow dung, thermostable.

INTRODUCTION

The enzyme cellulase deconstructs plant structural material by breaking down their cell walls. Cellulase is secreted by a great diversity of microorganisms during their growth on cellulosic materials (Rajeeva *et al*., 2015). Thermostable alkaline cellulases is used in paper industry to increase the brightness, in food industry to improve the digestibility of animal feed and to clarify the juices (Kuhad *et al*., 2011) and in textile industry for wet processing (Shah 2013).

Cellulose is considered as the most important natural renewable resource for bioconversion, and is naturally present in the environment in various decomposable sources. This organic matter is decomposed by fungi, bacteria and actinomycetes, which are also naturally available in the environment (Immanuel *et al*., 2006). Among these microorganisms, bacteria are more capable of decomposing cellulose by the secretion of cellulase enzyme. Previously, different genera of bacteria have been reported for cellulase production. These cellulase producing bacteria include *Bacillus, Clostridium, Cellulomonas, Rummminococcus, Alteromonas, Acetivibrio* etc. (Rajeeva *et al*., 2015). Applications of cellulases extend to wine, beer and fruit juice production. Due to increased concern about the greenhouse effect, depleting oil reserves and rising global oil prices, as well as the focus on utilising renewable fuels such

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as bioethanol, cellulase enzymes have become quite important to keep the environment active and interactive (Mohanappriya & Kapilan, 2018). When these cellulase producers are isolated from naturally occurring sources under suitable conditions, they could be utilised in industries that use cellulase. Therefore, the study was aimed at isolating a thermostable alkaline cellulase producer from various natural sources (goat excreta, cow dung, tropical soil and organic matter) and to determine the kinetic properties and stability of the enzyme.

METHODOLOGY

Sources of bacterial strains

All the bacterial strains were isolated from goat excreta, cow dung, tropical soil and organic matter under required conditions from different areas of the Northern region of Sri Lanka. Isolates were further screened on the basis of carboxymethyl cellulase produced in liquid medium. Based on the amount of cellulase produced after 24 h of fermentation, the bacterial strain that grew on cow dung was selected as the best cellulase producing strain.

Chemicals and media

Chemicals used were from standard sources (Sigma Chemical Company, USA; BDH Ltd., England; Oxoid, England) unless otherwise stated. The activation medium contained (gL^{-1}): cellulose 20.0 and nutrient broth 28.0. Single colonies of the selected bacteria were obtained by cultivating them in nutrient agar (NA) medium at 37 ^{o}C for 24 h. The bacterial cells grown on the slants were activated in the activation medium (2 loops/10 mL), and incubated in a shaker water bath at 40 ^{o}C and 100 rpm for 48 h.

Cultivation of the strains

The strains from the stock cultures were streaked on solid NA and incubated at 37 ^{o}C. Grown cultures were cultivated in liquid LB broth media and spun in an orbital shaker at 100 rpm (Singh *et al*., 1998). In shaker flask experiments, media volume to the flask volume ratio was maintained as 1:4. Organisms from multiple sources were transferred on to the NA plate under sterile conditions and kept in the incubator at 37 ^{o}C for 48 h. Different colonies were then transferred on to new NA plates in order to get pure strains.

Production of cellulase and measurement of cellulase activity

The isolates, selected on the basis of plate staining method were grown in 50 mL of fermentation medium at pH 7.0 containing the following components (g/L): CMC (10.0), K_2HPO_4 (1.0), KH_2PO_4 (1.0), $MgSO_4 \cdot 7H_2O$ (0.2), NH_4NO_3 (1.0), $FeCl_3 \cdot 6H_2O$ (0.05), $CaCl_2$ (0.02), and yeast extract (5.0). To the fermentation medium, 20 % (v/v) inoculum was added and incubated at 40 ^{o}C in a rotatory shaker (100 rpm). Samples were taken after 42 h and centrifuged. The supernatant was used as the crude cellulase source. Screening was done by performing the cellulase enzyme activity assay to the supernatant collected from the liquid media of each selected bacterium from the culture that was allowed to ferment for 24 h (Vipul *et al*., 2012).

Morphological and biochemical characterisation of the isolate

Morphological and biochemical properties of the isolates were determined as described in the Bergey's Manual of Systematic Bacteriology (Bergey *et al*., 2001). Cell morphology of the selected isolate was observed by simple staining, Gram staining, endospore staining, and urease test done as per the standard protocol (Cappuccino & Sherman, 2004). The selected bacterial strain was subjected to a series of cultural, biochemical and morphological analyses such as colony size, shape and colour, single cell shape, spore formation, catalase test, oxidase test, triple sugar iron agar test and lactase fermentation test in order to identify the genus and species of the strain (Singh *et al*., 1998). Catalase activity was determined by adding a few drops of 3 % (v/v) H_2O_2 to 5 mL of 16 h young culture. Nitrate agar slants were used to test nitrate reducing property of the selected strain. BHM amended with starch was used for amylase activity determination. Triple sugar iron (TSI) slants containing three sugars, namely, glucose, lactose, and sucrose, were used for acid and H_2S production test. Acid production after carbohydrate fermentation was detected by the visible change in colour from red to yellow. The temperature tolerance test was performed by growing the strain in nutrient broth and incubating at the temperatures ranging 20 – 50 ^{o}C.

Characterisation of the strain by molecular means

Pure culture of the selected bacterial strain was grown overnight on nutrient broth and DNA extraction

was done by cell lysis method using a kit (QIAGEN Inc., Mississauga, ON, Canada). 16S rDNA was amplified by thermocycler using the primers; forward: 5' AGAGTTTGATCCTGGCTCAG 3', Reverse: 5' TACCTTGTTACGACTT 3'. The amplified 16S rDNA PCR product was sequenced using automated sequencer. Sequence similarity search was done for the 16S rDNA sequence using online search tool BLAST (*http://www.ncbi.nlm.nih.gov/blast/*). The unknown organism was identified using the maximum aligned sequence through BLAST (Claudio *et al*., 2002).

Kinetic studies

Activity of cellulase with time

Cellulose (20 gL^{-1} pH 9.0; 20 mL) was taken in a 100 mL conical flask and pre-incubated at 45 °C for 3 min in a water bath. Similarly, a boiling tube containing crude cellulase solution (20 mL) was also pre-incubated. Pre-incubated soluble cellulase (20 mL) and cellulose (pH 9.0; 20 mL) were mixed together in 1:1 ratio and incubated for 4 h at 45 °C. At definite time intervals (0, 2, 5, 8, 10, 15, 20, 25, 30, 45, 60 and 75 min) 1 mL of the sample was withdrawn from the reaction mixture and glucose production was monitored.

Effect of temperature

The effect of temperature on crude cellulase activity was determined by incubating the enzyme for an optimised amount of time with 0.25 mL of soluble cellulose (50 gL^{-1}) at pH 9.0 at different temperatures.

Effect of pH on cellulase activity

The effect of pH on cellulase activity was measured by preparing 50 gL^{-1} soluble cellulose in buffers at different pH values (pH 5 – 6: citrate-phosphate buffer; pH 8.0: Tris-aminomethane buffer; pH 9.0: glycine-NaOH buffer and pH 10 to 11: phosphate buffer) and incubating at optimised temperature (45 °C).

Effect of substrate concentration

Different concentrations of the cellulose solution were prepared (8 to 10 gL^{-1}) in 0.01 M Tris buffer at optimum pH and were allowed to react with the diluted enzyme at optimum conditions (45 °C, pH 9.0, 10 min). Then the enzyme activity was measured.

Effect of pH on the stability of cellulase

Effect of pH on the stability of the enzyme at different pH values was also studied by incubating the enzyme at different pH values ranging from 8.0 to 11.0 at 45 °C and the residual activity was determined using 50 gL^{-1} cellulase under optimised conditions (at 45 °C, pH 9.0). All the experiments were done in triplicate and the average was used for analysis.

Analytical methods

Cellulase activity was assayed according to the modified method described by Ghose (1987). The glucose produced was measured by dinitrosalicylic acid (DNSA) method. One unit of cellulase activity is defined as the amount of enzyme that produces 1 µmol of reducing sugar in 1 min at pH 5.0 and 50 °C with 10 mL (1g/100 mL) cellulose. The values of enzymatic activity were expressed as U/mL. Reducing sugar was measured by DNS method (Miller, 1959). Based on the amount of cellulose production after 24 h of fermentation, the best cellulose producer was selected. The following formula was used:

Cellulase activity = [(0.5 $/Standard\ reading$ of OD at 0.5 $mg\ glucose$) x OD of test x (1/$volume of supernatant$) x (1 / $time$) x (1000/ 180)]

Statistical analysis

All experiments were carried out in triplicate and the mean and standard deviation values were used to plot the graphical representations. The entire set of kinetic experiments were also done in triplicate and the values were from 3 representative experiments. Statistical analyses were performed using R2.15.3 (R Development Core Team, 2010). The data were analysed using ANOVA. Determination of significant differences at $p \leq 0.05$ was estimated by performing Tukey's multiple comparison test.

RESULTS AND DISCUSSION

Isolation of cellulase producers

Bacterial strains, which produce cellulase were isolated from goat excreta, cow dung, tropical soil and organic matter by using selective carboxy methyl cellulose (CMC) containing NA medium. Among the total of 42

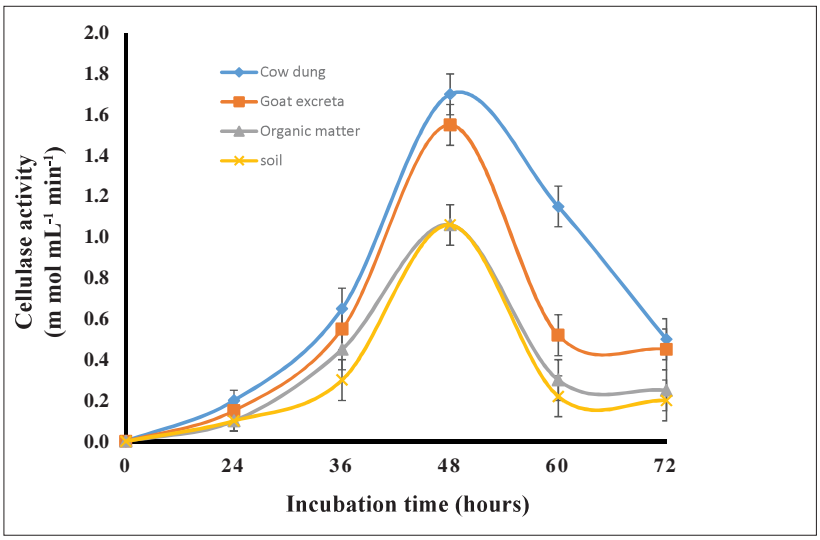

Figure 1: Cellulase production by the best substrate-specific isolates extracted from different sources: cow dung, goat excreta, organic matter and tropical soil

isolates, 17 cellulose hydrolysing microorganisms were screened on the basis of plate staining method. All the isolates showed clear zones around colonies after staining the plates with congo red and destaining with 1M NaCl. However, the plate-screening method is not quantitative because of the poor correlation between enzyme activity and the colony to clear zone ratio.

Morphological and biochemical characterisation

All the strains started to show cellulase activity at 24 hours at all the tested temperatures when the pH was maintained at the initial level. According to a previous study, cellulase activity was obtained at 16 hours for *Bacillus brevis* (Singh *et al*., 1998). Qualitative assay was conducted for screening cellulase activity. Based on the amount of cellulase production after 24 hours of fermentation, the best cellulase producer (B2) that was growing on cow dung was selected. Among the 15 bacterial samples isolated from cow dung, 7 strains were confirmed as cellulase producers. Among them, the best cellulase producing strain was selected for further studies (Figure 1).

Characterisation of the bacterial strain by molecular means

Based on the culture and morphological studies, the genus of the selected strain (B2) was suspected to be *Bacillus* since it showed positive results to Gram staining, spore formation, motility, catalase test and triple sugar iron agar test (Table 1). Species-specific DNA sequences could be used to find different microbial species (Basavaraj *et al*., 2014). DNA from the strain B2 was isolated and the 16S rDNA was amplified and

Table 1: Morphological, microscopic and biochemical characterisation of the selected strain

Isolated strain B2	Morphological, microscopic, biochemical and cultural characteristics
Form	Circular
Elevation	Convex
Margin	Entire
Opacity	Opaque
Diameter after 40 h (in mm)	1.5–2.0
Colour	White
Surface	Moist, shiny
Grams staining	+
Shape of vegetative cell	Rod shape
Spore formation	+
Motility	+
Growth in air	+
Catalase production	+
Triple sugar iron agar test	+
Lactose fermentation	-
NO_3 reduction test	+
Urease test	+
Growth at 50 °C	+

sequenced. The BLAST analysis of the strain using its 16S rDNA sequence data showed that the strain CEL PTK1 had the highest homology (100 %) with *Bacillus cereus* (GenBank accession no AF290555).

Kinetic studies of the cellulase

Activity of cellulase with time

Cellulase showed zero order kinetics for 20 minutes (Figure 2). Therefore, the incubation time for the kinetic studies was fixed as 10 minutes for the following experiments.

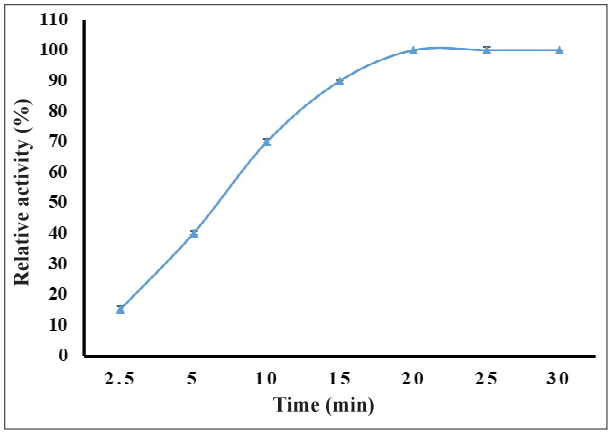

Figure 2: Production of glucose from cellulose (50 gL^{-1}) by the crude cellulase enzyme from *Bacillus cereus* at pH 9.0 at 45 ^{o}C

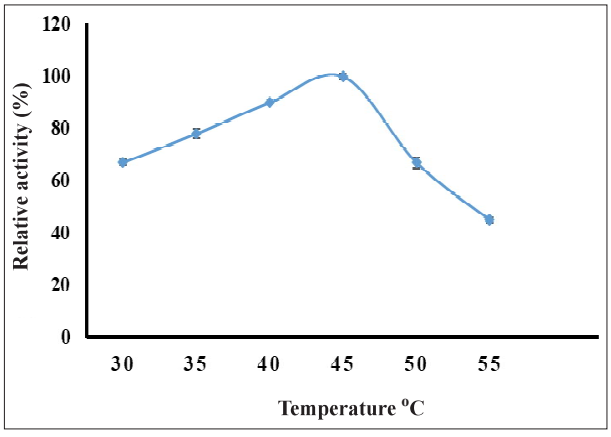

Figure 3: Effect of temperature on the activity of crude cellulase enzyme from *Bacillus cereus* at pH 9.0 with cellulose (50 gL^{-1})

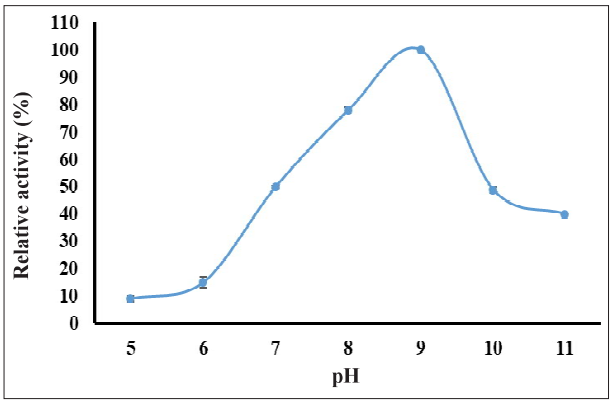

Figure 4: Effect of pH on the activity of cellulase enzyme from *Bacillus cereus* at 45 ^{o}C with cellulose (50 gL^{-1})

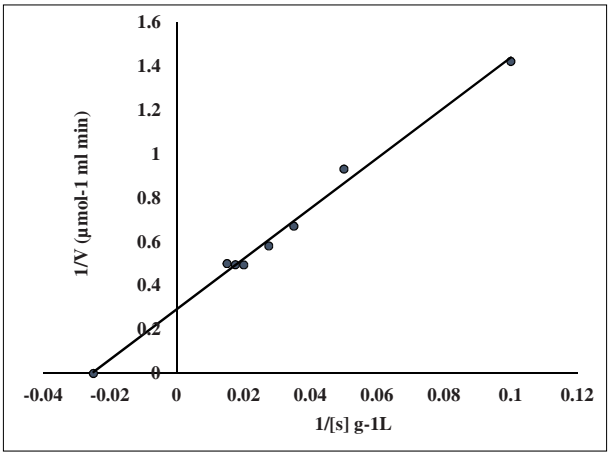

Figure 5: Lineweaver-Burk plot of the crude cellulase from bacterial strain *Bacillus cereus* isolated from cowdung at pH 9.0 and 45 ^{o}C with different concentrations of cellulose substrate

Effect of temperature

Cellulolytic activity of the cellulase was assayed at different temperatures ranging from 20 – 55 ^{o}C at pH 9.0. The activity of cellulase increased up to 45 ^{o}C and further increase in temperature lowered the enzyme activity (Figure 3). Therefore, this moderately higher temperature (45 ^{o}C) could be suggested for use in commercial applications.

Effect of pH

When the pH was increased from 5.0 to 11.0, the activity of cellulase increased up to pH 9.0 and further increase of pH lowered the enzyme activity (Figure 4). Cellulase

enzyme from the *Bacillus* isolate showed activity between pH 5.0 to 11.0. However, this enzyme showed 75 % of its original activity at pH values between 7.0 and 10.0. Since the highest cellulase activity was obtained at pH 9.0, this pH value was selected for further studies.

Effect of substrate concentration

When the substrate concentration was increased from 10 to 80 gL^{-1} at pH 9.0, the activity of cellulase increased up to 50 gL^{-1} and reached the maximum velocity at that level (Figure 5). Michaelis constant for the crude enzyme to soluble cellulose was 38.60 $g^{-1}L$ and V_{max} was 3.322 $\mu molmL^{-1}min^{-1}$ at pH 9.0 and at 45 ^oC (Figure 5). Therefore, 50 gL^{-1} substrate was used for the assay for further studies of enzyme activity.

Effect of pH on the stability of cellulase

When the crude enzyme solution at pH 8.0 –11.0 was pre-incubated at 45 ^oC, the enzyme activity decreased with time. At pH 8.0, the crude enzyme retained 74.7, 61.2, and 6.1 % of the activity at 30, 60 and 75 minutes, respectively at 45 ^oC. At pH 9.0, the crude enzyme retained 91.26, 68.5 and 14.9 % of the cellulase at 30, 60 and 75 minutes, respectively. At pH 10.0, the crude enzyme retained 40.5, 24 and 0 % of the cellulase at 30, 60 and 75 minutes, respectively. Thus, the half-life of cellulase was 18.5, 27.3 and 7.5 minutes at pH 8.0, 9.0 and 10.0, respectively at 45 ^oC indicating that cellulase is more stable at pH 9.0. It is also important to note that the enzyme showed optimum activity at pH 9.0.

CONCLUSION

Strain B2 was selected as the best thermostable alkaline cellulase producer among the isolated strains, as it gave the highest cellulase activity in alkaline pH and at high temperatures and this activity was obtained in a short time of fermentation than all the other strains. According to the morphological and biochemical tests and 16S rRNA sequencing, the selected strain was identified as *Bacillus cereus* with the GenBank accession number AF290555. Cellulase from *B. cereus* showed zero order kinetics for 20 minutes and the pH optimum for the cellulase activity was 9.0 at 45 ^oC and the temperature optimum was 45 ^oC at pH 9.0. Michaelis constant for the cellulase enzyme to soluble cellulose was 38.60 $g^{-1}L$ and V_{max} was 3.32 $\mu molmL^{-1}min^{-1}$ at pH 9.0 and at 45 ^oC. This cellulase enzyme was stable for more than an hour at 45 ^oC and at pH 9.0.

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REFERENCES

Basavaraj I., Patagundi C.T., Shivasharan & Kaliwal B.B. (2014). Isolation and characterization of cellulase producing bacteria from soil. *International Journal of Current Microbiology and Applied Sciences* **3**(5): 59–69.

Boone D.R., Garrity G.M., Castenholz R.W., Brenner D.J., Krieg N.R. & Staley J.T. (2001). Genus *Bacillus*. *Bergey's Manual of Systematic Bacteriology*, pp. 21–128. Springer, New York, USA.
DOI: https://doi.org/10.1007/978-0-387-21609-6

Cappuccino J.C. & Sherman N. (2004). *Microbiology - A Laboratory Manual*, 7th edition. Pearson Education Publication, New Delhi, India.

Ghose T.K. (2016). *Measurement of Cellulase Activities*. International Union of Pure and Applied Chemistry Standards Online. New Delhi, India.
DOI: https://doi.org/10.1515/iupac.59.0006

Immanuel G., Dhanusha R., Prema P. & Palavesam A. (2006). Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. *International Journal of Environmental Science and Technology* **3**(1): 25–34.
DOI: https://doi.org/10.1007/BF03325904

Kuhad R.C., Gupta R. & Singh A. (2011). Microbial cellulases and their industrial applications. *Enzyme Research* **2011**: Article ID 280696.
DOI: https://doi.org/10.4061/2011/280696

Miller G.L. (1959). Use of Dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry* **31**: 426–428.

Mohanappriya S. & Kapilan R. (2018). Isolation of efficient cellulase producing *Aspergillus unguis* UCSC324 and determination of the kinetic properties of its crude cellulose. *AGRIEAST: Journal of Agricultural Sciences* **12**(2): 35–48.
DOI: https://doi.org/10.4038/agrieast.v12i2.58

Rajeeva G. & Soni T. (2015). Isolation, production, purification and characterization of an organic-solvent-thermostable alkalophilic cellulase from *Bacillus vallismortis* RG-07. *BMC Biotechnology* **15**(19): 1–12.
DOI: https://doi.org/10.1186/s12896-015-0129-9

Sacchi C.T., Whitney A.M., Mayer L.W., Morey R., Steigerwalf A., Ariana B., Weyant R.S. & Popovic T. (2002). Sequencing of 16S rRNA gene: a rapid tool for identification of *Bacillus anthracis* bioterrorism-related anthrax. *Emerging Infectious Disease* **8**: 1117–1123.
DOI: https://doi.org/10.3201/eid0810.020391

Shah S.R. (2013). Chemistry and applications of cellulase in textile wet processing. *Research Journal of Engineering*

Sciences **2**(7): 1–5.

Singh V. & Kumar A. (1998). Production and purification of an extracellular cellulase from *Bacillus brevis vs-1*. *International Union of Biochemistry and Molecular Biology Life* **45**(3): 443–452.

DOI: https://doi.org/10.1080/15216549800202832

Vipul V., Alpika V. & Akhilesh K. (2012). Isolation and production of cellulase enzyme from bacteria isolated from agricultural fields. *Advances in Applied Science Research* **3**(1): 171–174.