COMPREHENSIVE ELUCIDATION OF STRUCTURAL AND COMPOSITIONAL CHANGES IN COTTON STALKS UNDERGOING BIODEGRADATION BY POTENT CELLULOLYTIC BACTERIA

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The present study aimed the exploration of cellulose degrading bacteria as cotton stalk degrader and to characterize the thus treated cotton stalk at compositional and structural levels. The bacterial strain *Priestia megaterium*, coded as NAU-WP-1, was identified by its microbial and molecular characteristics and revealed a cellulolytic index of 2.23, CMCase of 0.223 IU/min/mL and FPase of 0.098 IU/min/mL with carboxymethyl cellulose (CMC) medium for 72 h at 30 ± 0.2 ºC. The cellulolytic potential of the strain was studied to evaluate the degradation of cotton stalk. Biodegradation studies of cotton stalk revealed the maximum CMCase (0.563 IU/min/mL) and FPase (0.1159 IU/min/mL) activity on the 30th and 25th days, respectively. Composition analysis of *Priestia megaterium* NAU-WP-1 treated cotton stalk showed reduction in weight (7.68%), moisture (5.75%), cellulose (43.70%), nitrogen (10.26%), phosphorus (38.64%), potash (9.29%) and ash (7.79%). Fourier transform infrared spectroscopy (FT-IR) analysis was performed to confirm the structural changes appearing in the microbially treated cotton stalk sample on the 30th day compared to the control.FT-IR data revealed that the peaks observed in the control sample at 3361 cm^{-1} (OH stretching), 2916 cm^{-1} and 2849 cm^{-1} (C-H stretching), 1078 cm^{-1} (C-O stretching), and 1050–1150 cm^{-1} (C-O-C) either shifted, downward-shifted, or disappeared completely in the microbially treated sample. These changes indicated modifications in hydroxyl groups, breakdown of carbon-hydrogen bonds, and alterations in ether linkages within the cellulose structure. Structural modification and hydrogen bond cleavage were identified as the predominant mechanisms behind the degradation of cotton stalks by *P. megaterium* NAU-WP-1. Furthermore, UV-spectra analysis showed a significant reduction in the peak at 275 nm, corresponding to the intact cellulose structure in the treated sample, compared to the control, which further confirmed the enzymatic breakdown of cellulose by the bacteria. Hence, this study highlights *Priestia megaterium* NAU-WP-1 as a potential cotton stalk degrader and underscores the need for further comprehensive studies to explore its application in agricultural residue management practices.

Keywords: cellulolytic, cotton stalk, CMCase, FPase, *Priestia megaterium*

INTRODUCTION

Agricultural biomass is the richest and most replenishable source of cellulose. Among various agricultural residues, cotton stalk possesses high structural complexity and is the richest source of cellulose. The breakdown of the microcrystalline structure of the cellulose remains a significant challenge for proper utilization of agricultural cellulosic biomass.[1] Further, hydrolysis is a key rate limiting step during the digestion of cotton stalk and thus its pretreatment is essential. Existing physiochemical pretreatment technologies, such as steam explosion, acid or alkali treatment, oxidation, and their combinations, are based on high-energy, corrosion-resistant and high pressure parameters. These physical and chemical treatments require high-throughput equipment, making them expensive.[2] In recent years, alternative biological technologies have shown great promise.

Biological methods based on utilizing microbes have gained attention due to their high potential to convert the complex structure of cellulose into a simpler form. The cellulose hydrolytic enzymes called “cellulase complex system” produced by a wide range of microbial communities have inbuilt

ability to break down the microcrystalline cellulose, as well as lignocellulosic structure. Agricultural residues and other biomass wastes, such as wheat straw, sorghum straw, bagasse, sawdust *etc*., are often treated with cellulose degrading microbes in order to produce industrially important cellulase enzyme using solid state fermentation.[3]

Generally, fungal strains are predominantly used to treat agricultural biomasses due to their high production of the cellulase complex.[5] However, Zheng *et al.* stated that bacterial consortia are much more potent to degrade cellulose and hemicelluloses than fungal strains.[4] Further, the characteristics of bacteria, such as rapid growth rate, high genetic variability, adaptability and easy bioformulation preparation, make them more attractive to treat agricultural waste.[6] Presently, the isolation, screening and exploitation of bacterial strains in order to degrade agricultural biomass have gained increasing interest.[7] Previously, Velmourougane *et al.* used a bacterial consortium to prepare compost from cotton stalk, which was used as a substitute for soil.[8] An array of research work, including the use of bacterial cellulase complex or consortia, has been conducted to convert cotton stalk into valuable products. However, the biochemistry behind the breakdown of the complex structure of cotton stalk through bacteria is rarely studied. Therefore, the present study elucidated the compositional and structural changes of cotton stalk treated with a potent cellulose degrading bacterial strain.

Samples like soil, manure, gut microflora of insects, and feces of the ruminants have been used to isolate cellulose degrading bacteria. Additionally, rotten or decayed wood is also the richest source for isolation of cellulose degrading bacteria.[9] Our group is involved in designing various chemical and biological routes for the dissolution and regeneration of cellulose.[6,10–15] In the present work, we aim to isolate, characterize and identify cellulose degrading bacteria from decayed wood, seeking for a potential bacterial strain to degrade cotton stalk. The compositional and structural changes occurring in bacterially degraded cotton stalk were examined. Cellulose degrading bacteria were isolated and screened through qualitative and quantitative assays. A total of eight bacteria were further identified as cellulose degraders, out of the 18 bacterial strains. Out of the 8 (eight) cellulose degraders, *Priestia megaterium* NAU-WP-1 was found to be the most effective, as confirmed by its significant cellulolytic index (2.23 ± 0.020), CMCase (0.223 ± 0.0012 IU/min/mL) and FPase (0.098 ± 0.0006 IU/min/mL) activities at room temperature (30 ± 0.2 °C). Using these cellulolytic bacteria, the biodegradation of cotton stalks (*Gossypium hirsutum* L.) was carried out and total cellulase activity (FPase activity) and endoglucanase activity (CMCase) were analyzed. FT-IR and UV spectral data indicated that the peak intensities corresponding to the lignocellulosic structure were dramatically shifted, diminished or totally disappeared in the case of the bacteria treated cotton stalk sample, compared to the control, which confirmed the breakdown of cotton stalk by bacteria. Likewise, compositional analysis data revealed 43.70% reduction of cotton stalk cellulose by our *P. megaterium* NAU-WP-1 strain, strongly emphasizing its potential as an efficient cellulose degrader.

EXPERIMENTAL

Isolation and screening of cellulose degrading bacteria

Decayed wood samples were collected from the farm of the Main Cotton Research Station, Athwa farm, Navsari Agricultural University (NAU), Surat (21°10'20.2"N 72°48'03.9"E). The standard microbiological method was used to isolate bacterial colonies on nutrient agar media. Screening of cellulose degrading bacteria was carried out through qualitative and quantitative assays. Qualitative screening was done by spot inoculation of each purified bacterial colony on carboxymethyl cellulose (CMC) agar plates as per the method of Kameshwar and Wensheng *et al*.[16] Quantitative assay was performed by analysis of CMCase and FPase produced by the bacteria in the CMC broth. Crude enzyme activity of CMCase (IU/min/mL) and FPase (IU/min/mL) was assessed as per the method of Sherief *et al.*[17]

Identification of the potential cellulose degrader

Identification of the bacteria was carried out using microbial and molecular attributes. The KB002 Hi-Assorted Biochemical test kit was used for biochemical characterization of bacteria; while gram reaction and nutrient media were used to identify the morphology and cultural characteristics of the bacteria.

Molecular identification was done by extracting the pure and intact bacterial genomic DNA as per the method reported by Nakada *et al.*[18] Amplification of 16S rDNA was performed using universal primers 27F (5'-CCAGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTT GTTACGACTT-3').[19] The amplified PCR product was further purified by salt-precipitation, then subjected to cycle sequencing using BDT v3.1 chemistry and subsequently sequenced on an ABI 3500XL Genetic Analyzer. Sequencing services

are taken from Hi-Gx360® Solutions, HiMedia Laboratories Pvt Ltd. The consensus sequence was subjected to a database search against an appropriate database using the BLAST tool.[20] For the phylogenetic analysis, up to 10 closest-neighbour sequences belonging to different taxa from amongst the top 1000 hits with the highest similarity in the search results were retrieved from the database and aligned using the MUSCLE aligner.[21] The multiple sequence alignment was manually inspected and used to produce a consensus phylogram using an appropriate algorithm with 1000 iterations using MEGA11 (Molecular Evolutionary Genetic Analysis, version 11) software.[22]

Cotton stalk biodegradation studies

Cotton stalk preparation

Cotton stalk (*Gossypium hirsutum* L.*)* was collected from the field of the Main Cotton Research Station (MCRS), Navsari Agricultural University (NAU), Athwa Farm Surat, Gujarat, India. Cotton stalk was sun-dried, crushed and sieved to 3 mm particle size, using a mill, and stored in air-tight containers for its further use.[23]

Bacterial inoculum preparation

Enrichment of the bacterial culture was done using 10 mL of sterile minimal broth embedded with 1% of cotton stalk as per the method of Zheng *et al*.[24] The broth was kept at room temperature (30 ± 0.2 ºC) for ten days.

Enzyme production during cotton stalk biodegradation

Cotton stalk biodegradation was studied by inoculation of 10 mL of enriched inoculum into 90 mL of sterile minimal medium, containing 10 g of cotton stalk powder.[24] Control flasks were kept without inoculation of the bacteria. All flasks were kept in static condition at 30±1 ºC. At time intervals of five days, samples were withdrawn from both test and control flasks and filtered using filter paper (size – 9.0 cm diameter; Hi-media). Filtrate was used to analyze total cellulase activity (FPase activity) and endoglucanase activity (CMCase) as per the method of Ghose (1987). The amount of reducing sugar released was estimated using the dinitrosalicyclic acid (DNS) method.[25] One unit (IU) of FPase and CMCase activity was defined as the amount of enzyme releasing one µmol of reducing sugar per min.

Composition change of cotton stalk before and after degradation

The percent change in the amount of cellulose, nitrogen, phosphorus, potash, weight loss, moisture and ash content of the cotton stalk was assessed as per the method of Thimmaiah, to determine the compositional analysis of cotton stalk.[26]

Fourier transform infrared spectroscopy (FT-IR) and UV-vis spectroscopy

The Fourier transform infrared (FT-IR) spectra of the control and bacteria treated cotton stalk samples were recorded using a Shimadzu FT-IR-8400S spectrometer, in the range of 4000–400 cm^{-1}, with a resolution of 4 cm^{-1}, and an average of 40 scans per sample. This analysis aimed to identify the changes of functional groups occurring through the degradation process. Additionally, the degradation of the cotton stalk was assessed using UV-vis spectroscopy, recorded on a Cary-50 UV-vis spectrophotometer from Agilent, within the wavelength range of 200–800 nm, with a cuvette path length of 1 cm and an appropriate solvent as a reference.

Statistical analysis

The one-way analysis of variance (ANOVA) and complete randomized design with factorial concept was used. The critical difference (CD) among the variance was calculated at $P \leq 0.05$.[27] The results are expressed as mean with standard deviation (mean ± SD) or standard error (mean ± SE).

RESULTS AND DISCUSSION

Isolation and screening of cellulose degraders

A total of 18 (NAU-WP-1 to NAU-WP-18) bacteria were isolated on nutrient agar from the decayed wood sample. Among these 18, eight isolates were identified as cellulose degraders based on qualitative and quantitative assays. Among the eight bacterial isolates, NAU-WP-1 was observed to have a significant cellulolytic index (2.23±0.020), and CMCase (0.223 ± 0.0012 IU/min/mL) and FPase (0.098 ± 0.0006 IU/min/mL) activities at room temperature (30 ± 0.2 ºC) after 72 h in CMC medium during qualitative and quantitative screening, respectively. Previously, Paudel and Qin[9] isolated 17 cellulose degrading bacteria from a rotting wood sample, whereas Roy *et al.* isolated 20 cellulolytic bacteria and fungi from partially decomposed cellulose rich substrates, like residues of groundnut, rice and decayed wood samples.[28]

Identification of NAU-WP-1 strain

Morphological and culture characteristics revealed that the strain NAU-WP-1 was a gram positive bacterium, with a long chain, and formed large, round, opaque, irregular and non-pigmented colonies on nutrient agar plates. Biochemically, the strain showed positive results with lysine, ornithine, nitrate reduction, methyl red, esculin, cellobiose, saccharose, glucose and lactose, oxidase and catalase; while negative results with ONPG, urease, phenylalanine deaminase, H_2S

production, citrate utilization, Vogus-Proskauer (VP) test, indole, malonate, arabinose, xylose, adonitol, rhamnose, melibiose, and raffinose. Microbiologically, the characteristics were similar to those of the *Bacillus* genus.

Furthermore, to determine the identity of NAU-WP-1, a fragment of 16S rDNA was sequenced. The sequence, 1444bp long, was compared to the available sequences in the GenBank database using the BLAST browser (NCBI), followed by alignment of sequence using multiple alignment software program Clusal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 11.[22] The sequence of the PCR product was deposited in NCBI (Accession no. OR880055.1).

Based on the data, the bacterial strain NAU-WP-1 showed 100% similarity to the strain *Priestia megaterium* that was previously known as *Bacillus megaterium* (Fig. 1). In accordance with microbial and molecular data, the strain identified as *Priestia megaterium* NAU-WP-1. Our findings supported the report of Long *et al.* and Roy *et al.*, who prepared an efficient cellulose degrading bacterial consortium, which also included *Priestia megaterium*, and they found it to be one of the potential cellulose degrader strains.[28,29]

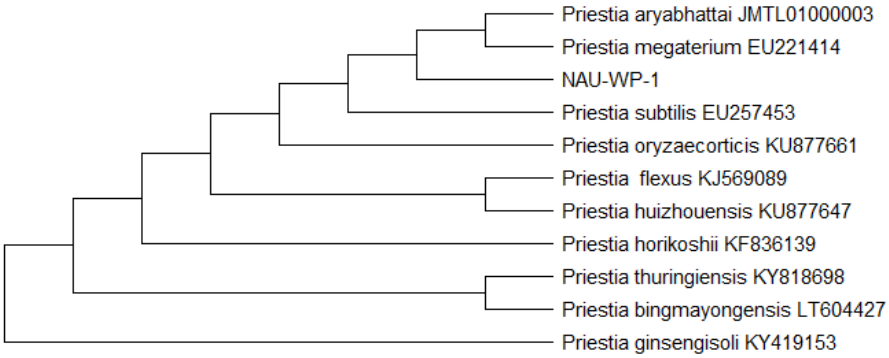

Figure 1: Phylogenetic relationship of NAU-WP-1 with other bacterial sequences

Biodegradation studies

Enzymatic and compositional analysis

CMCase and FPase are pivotal enzymes produced by microbes during biodegradation of lignocellulosic material or agricultural waste. Therefore, the crude enzyme activity of CMCase and FPase was measured at five days intervals during the biodegradation studies of cotton stalk by the *P. megaterium* NAU-WP-1 strain. The data revealed that CMCase (IU/min/mL) and FPase (IU/min/mL) activities gradually increased over time. Furthermore, maximum activity of CMCase (0.563 IU/min/mL) and FPase (0.147 IU/min/mL) was observed on the 30th and 25th days of incubation, respectively (Fig. 2 (a)). The presence of enzymes during the studies indicated that *P. megaterium* NAU-WP-1 was able to degrade cotton stalk, indeed the optimization studies required to improve the activity of enzymes to accelerate the degradation process.

The compositional analysis of the bacteria treated and control cotton stalk samples was carried out after 30 days. Data on weight loss (%) indicated that the weight of cotton stalk decreased in the treated sample (80.00%) compared to control (86.77%) (Fig. 2 (b)). Yuan *et al*. loaded 4.0% of cotton stalk in the medium and observed 15.9% of weight loss with a microbial consortium (MC1) within 14 days. In the present study, a 7.68% of weight loss was observed within 30 days using a 10% of cotton stalk load.[30] Long *et al*. constructed a cellulose degrading consortium and achieved 26.00% weight loss of solid waste of spent mushroom residues after 20 days.[29,30] A significant weight loss of 7.68% with *P. megaterium* NAU-WP-1 alone indicated that it is a potent cellulose degrader. However, its cellulolytic activity can be further enhanced either by preparation of a consortium or by optimization studies.

Further, no drastic changes in moisture content were observed during the 30 days of the study. The moisture content (%) of the control and of the treated sample was 78.67 and 74.15, respectively (Fig. 2 (c)), which favours the degradation process and supports bacterial growth. The cellulose and ash content were 23.80% and 95.68% in the control sample; while 13.40% and 88.23% in the treated sample, respectively (Fig. 2 (d)). Thus, the cellulose and ash content were reduced to an extent of 43.70% and 7.79%, respectively. Wang *et al.*

used *A. fumigatus* Z5 inoculated lignocelluloses and cow dung to degrade rice husk and reported that the cellulose and hemicellulose content decreased after 28 days of incubation, but the ash and lignin contents increased, which suggested sufficient degradation of cellulose and hemicelluloses, but not lignin.[31] Further, in our study, the percent amounts of potash, phosphorus and nitrogen were recorded in the treated sample (2.93%, 0.27% and 2.10%), compared to the control (3.23%, 0.44% and 2.34%), (Fig. 2 (e)). These results indicate a 9.29%, 38.64% and 10.26% reduction in potash, phosphorus and nitrogen contents, respectively, in the bacteria treated cotton stalk, as compared to the control. Thus, the lower cellulose, potash, phosphorus, nitrogen and ash contents found in our present study indicated degradation of cellulose along with lignin. Moreover, the percent reduction of cellulose and phosphorus confirmed that *P. megaterium* NAU-WP-1 is a potent cellulose degrader, with great phosphorus utilization ability.

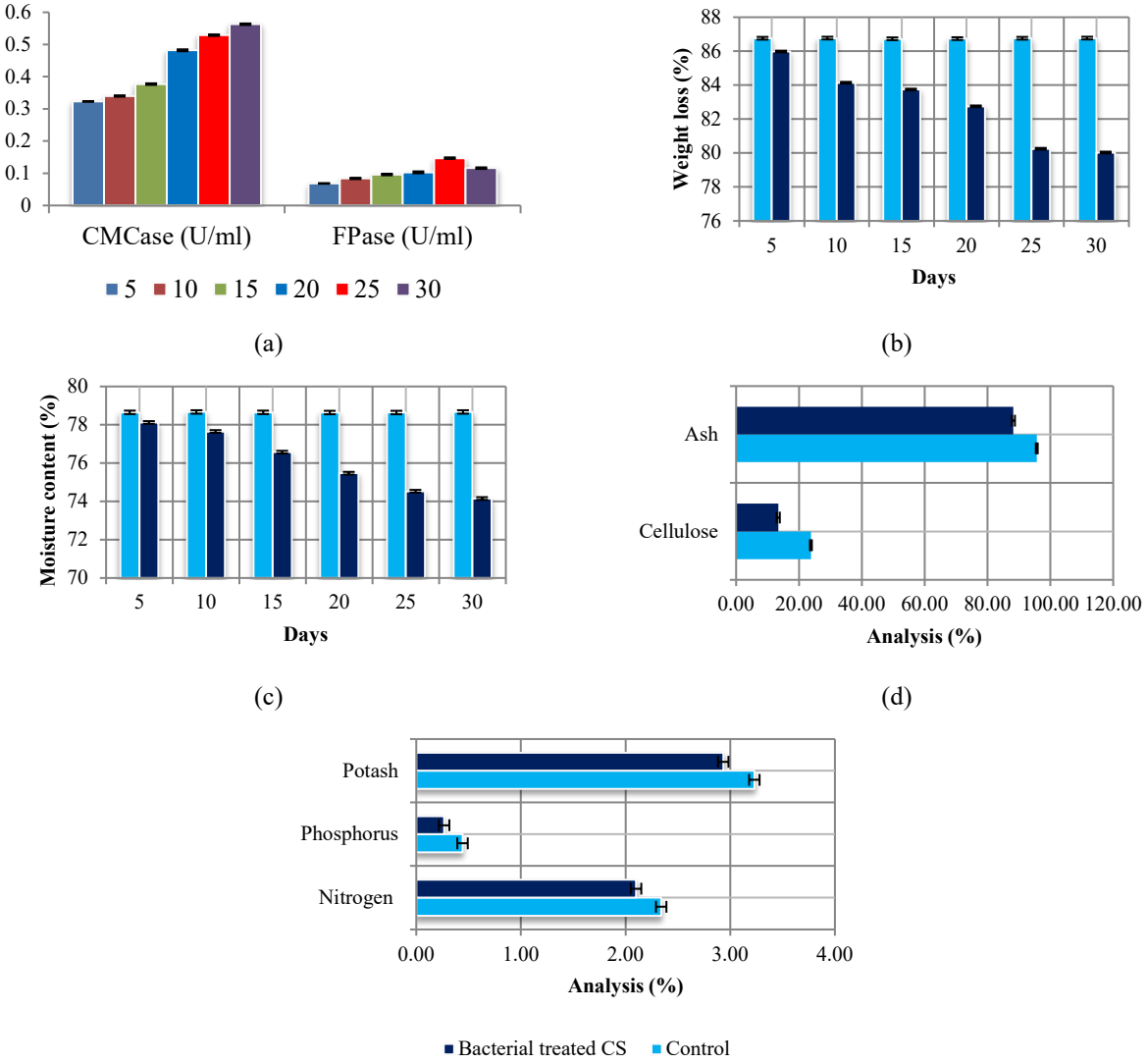

Figure 2: (a) Activity of CMCase (IU/min/mL) and FPase (IU/min/mL); Analysis of (b) weight loss (%), (c) moisture content (%) (d) ash and cellulose content (%) and (e) potash, phosphorus and nitrogen content (%) of cotton stalk

Cellulose degradation through FT-IR and UV-vis analysis

Cellulose degradation has been extensively studied using Fourier transform infrared (FT-IR) spectroscopy in various research studies. Margutti *et al*. explored the hydrolytic and oxidative degradation of cellulose paper, highlighting the importance of understanding the degradation

processes of cellulose-based materials. Ołdak *et al.* investigated the photo- and bio-degradation processes in polyethylene, cellulose, and their blends using ATR-FT-IR spectroscopy, emphasizing the role of FT-IR in studying degradation mechanisms.[32,33] The chemical components and the position of absorption peaks of the FT-IR spectra were assigned as per Haque *et al.*[34] The FT-IR spectra of the control and test cotton stalk sample are shown in Figure 3.

The comparison of FT-IR spectra between the control and the *P. megaterium* NAU-WP-1 treated samples reveals significant shifts in peak positions, indicative of cellulose degradation processes.[35,36] In the control, the peaks corresponding to OH stretching vibrations at 3361 cm^{-1} are observed, alongside the peaks related to C-H stretching vibrations at 2916 cm^{-1} and 2849 cm^{-1}, and C-O stretching vibrations at 1078 cm^{-1}. However, in *P. megaterium* NAU-WP-1 treated samples, there is a shift in the OH stretching peak to 3356 cm^{-1}, indicating potential modifications in the chemical environment of hydroxyl groups.[35,37] Notably, the absence of peaks in the vicinity of 2800-2900 cm^{-1} suggests potential degradation-induced changes in carbon-hydrogen bonds. The absence or emergence of peaks and shifts in peak positions provided insights into the extent and nature of cellulose degradation processes during the biodegradation process of cotton stalk.[38] Moreover, the peaks associated with the stretching vibrations of ether linkages (C-O-C) can typically be observed around 1050-1150 cm^{-1} in cellulose.[39] These peaks are prominent due to the presence of glycosidic linkages between glucose units in the cellulose polymer chain. In the spectrum of the control sample, a peak is observed at 1078 cm^{-1}, indicative of intact C-O-C stretching vibrations;[40,41] while in that of the sample treated with the *P. megaterium* NAU-WP-1 strain, the peak shifted to lower wavenumbers, suggesting a potential alteration in the chemical environment or bonding of the ether linkages within the cellulose structure. The downward shift in wavenumber may indicate changes in hydrogen bonding patterns or structural modifications induced by degradation processes. These data suggest that the degradation is mainly happening because of the hydrogen bond cleavage, as well as because of the structural modifications within the cotton stalks.

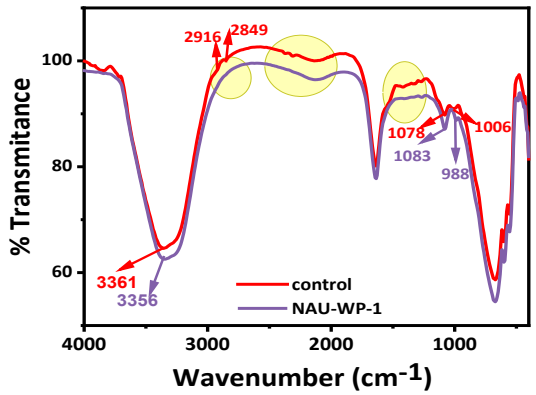

Figure 3: FT-IR analysis of cotton stalk treated by *P. megaterium* NAU-WP-1 and control sample

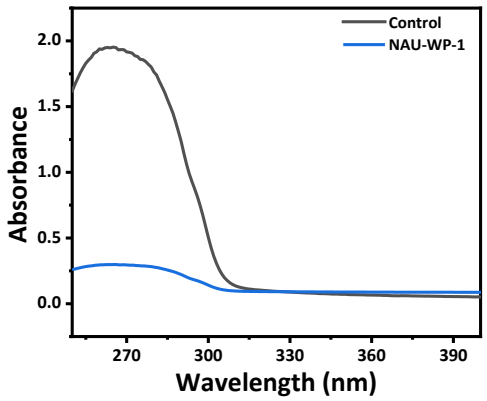

Figure 4: UV-vis absorption spectra of cotton stalk treated by *P. megaterium* NAU-WP-1 and control sample

The UV-vis absorption study (Fig. 4) provides clear evidence of cellulose degradation by *Priestia megaterium* NAU-WP-1 in comparison with the control sample. The control exhibits a distinct absorbance peak at approximately 275 nm, which is indicative of intact cellulose. This strong absorbance suggests that the cellulose structure remains largely undisturbed in the absence of bacterial treatment. However, the sample treated with *P. megaterium* NAU-WP-1 demonstrates a significant reduction in absorbance across the measured spectral range, particularly at 275 nm. This decline suggests that the cellulose polymer has undergone enzymatic breakdown, leading to the formation of smaller oligosaccharides, monosaccharides, or other degradation products that do not exhibit strong absorption in this region. The observed spectral changes confirm the cellulolytic activity of *P. megaterium* NAU-WP-1, reinforcing its potential application in biomass degradation and bioconversion processes. These findings align with previous reports on microbial cellulose degradation, further validating the

efficiency of *P. megaterium* NAU-WP-1 in lignocellulosic biomass processing.

The exploration of cellulose degrading microbes has been recognized as a promising eco-friendly approach to convert agricultural residue into value-added products. Previously, numerous studies have reported the isolation of cellulose degrading microbes from various soils, organic wastes, animal waste and gut microflora.[42,43] Among these, rotting or decaying wood is an outstanding source of potential cellulose degrading microbes.[9] Further, fungi are highly preferable in industries due to exclusive production of cellulase production, as compared to bacteria. Indeed, bacteria might be ideal candidates for cellulase production due to fast growth, easy culture and preparation of bioformulations.[44] Thus, the present study focused on a decayed wood sample to isolate cellulose degrading bacteria. A total of 18 bacteria were isolated and purified on nutrient agar plates and each was screened for cellulose degrading activity using carboxymethyl cellulose medium. The cellulolytic index (CI), CMCase and FPase activity were evaluated across eight potential cellulose degrading bacteria. Amongst them, bacterial isolate NAU-WP-1 was found to have remarkable CI and enzyme activity, and was identified through microbial and molecular characteristics. The microbial character of NAU-WP-1 was compared with the report of Hwang *et al.*[45] Based on the microbial and 16S rDNA sequence characteristics, the strain NAU-WP-1 was identified as *Priestia megaterium* NAU-WP-1 strain.

Microbial solid state fermentation is utilized to saccharify cellulosic materials, such as rice bran, wheat straw, sugarcane baggasse *etc.*[46,47] In the present study, *P. megaterium* NAU-WP-1 was assessed to degrade cotton stalk as cellulosic material, without any pretreatment and optimization studies. The results revealed that *P. megaterium* NAU-WP-1 strain showed CMCase and FPase activities during the periodic observation of cotton stalk degradation studies. The remarkable enzymes production by this strain during cotton stalk degradation indicated that it could be explored to produce cellulosic enzymes from cotton stalk waste. Further, compositional analysis of cotton stalk revealed that, on average, 5 to 45% reduction of weight, ash, cellulose, potash, phosphate and nitrogen content was achieved in *P. megaterium* NAU-WP-1 treated cotton stalk, compared to control, after 30 days, which indicated that the strain has the ability to break down the structure of cotton stalk. Moreover, the samples of cotton stalk were analyzed by FT-IR, which recorded shifting or disappearance of characteristic peaks; while UV analysis showed modification of the peak corresponding to an intact cellulose structure at 275 nm in *P. megaterium* NAU-WP-1 treated cotton stalk, compared to the control. These findings indicated that *P. megaterium* NAU-WP-1 has the ability to break down the cellulosic structure of cotton stalk.

The present study intended to achieve the degradation of cotton stalk by the strain *P. megaterium* NAU-WP-1, without pretreatment or consortia, led to the conclusion that the strain has the potential to break down the lignocellulosic waste and can be explored in further research for agricultural residue management.

CONCLUSION

Cotton stalk is the residue generated after harvesting of cotton crop and is rich in cellulose. The cellulase complex produced by the microbes is the hallmark of cellulose degradation. Thus, microbes producing the cellulase complex have attracted much attention in order to utilize them in the management of agricultural residues. Hence, the present study identified potential cellulose degrading bacteria, *Priestia megaterium* NAU-WP-1 isolated from a decayed wood sample, and investigated its use for cotton stalk degradation. The activity of enzymes (CMCase, FPase), compositional, and structural (FT-IR and UV) analysis of the bacteria treated cotton stalk sample, compared with the control, highlighted *Priestia megaterium* NAU-WP-1 as a potent cotton stalk degrader that could also be explored to produce cellulosic enzymes from cotton stalk waste. Comprehensive studies are required to develop a strategy for exploiting the strain in an economically beneficial management of cotton stalk.

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