



Developing Efficient Thermophilic Cellulose Degrading Consortium for Glucose Production From Different Agro-Residues

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In the present study, 11 cellulose degrading bacterial strains were isolated from water and soil samples of hot springs in the Chumathang village, Leh and Ladakh region, India. The isolated strains were identified as *Bacillus subtilis*, *Bacillus aryabhatai*, *Bacillus stratosphericus*, *Bacillus altitudinis*, and *Brevibacterium frigoritolerans* by biochemical and molecular approaches. All the strains were evaluated for the total cellulase, endoglucanase, exoglucanase, and β -glucosidase enzyme activities. On the basis of overall individual cellulose degrading enzyme activities, three strains were selected to develop consortium to enhance their cellulase enzyme activities. The potent cellulose degraders *B. stratosphericus* BHUJVPV-H5, *B. subtilis* BHUJVPV-H19, and *B. subtilis* BHUJVPV-H12 were selected for the consortium development. The effect of cellulase activities of bacterial strains were evaluated ranged up to 6.06 and 0.72 mg ml⁻¹ glucose by agro-residues of sugarcane bagasse and wheat straw, respectively, after 1 h of incubation. Total cellulase enzyme activity of consortia was recorded two times higher than the individual organisms. These strains can be used for enhancing bioethanol production from lignocellulosic biomass, which can consequently boost biofuel production industry.

Keywords: cellulase, endoglucanase, exoglucanase, β -glucosidase, sugarcane bagasse, wheat straw, glucose, consortium

HIGHLIGHTS

- *Bacillus* strains were isolated from hot spring's water and soil samples.
- Total cellulase enzyme activity was two times higher in the form of consortium.
- β -glucosidase activity was found to be high, with maximum 5.65 Uml⁻¹.
- Use of wheat straw and sugarcane bagasse showed good amounts of glucose release on hydrolysis.

INTRODUCTION

Cellulose degrading bacteria have three classes of enzymes which act synergistically on the cellulosic matrix to degrade it to monomer sugars. These classes of enzymes are endoglucanases, exoglucanases, and β -glucosidases. In nature, cellulose degrading bacteria (e.g., *Clostridium*, *Cellulomonas*, *Thermonospora*, *Bacillus*, *Bacteriodes*, *Ruminococcus*, *Erwinia*,

Acetovibrio, *Microbispora*, *Streptomyces*) have been isolated from different sources such as gut of termites, snail, bookworm, caterpillar (Gupta et al., 2011, 2012; Gupta and Verma, 2015), ruminants (bovine rum), soil samples (including deep subsurface soil), and human excreta (Robert and Bernalier-Donadille, 2003; Rastogi et al., 2009; Rawway et al., 2018). Lots of works in the past, since 1950s (Hungate, 1950), have been done to convert lignocellulosic biomass into bioethanol efficiently, but with little success (Gupta et al., 2012). Till date, no bacterial strain is reported with all three classes of cellulase enzymes needed for cellulose degradation. Hence, there is a need to explore novel bacterial strains for cellulose degradation into simple sugars which could be utilized as feedstock for bioethanol production.

Bacterial cellulases are preferred over fungal cellulases because of the following reasons. Their wide range of growth conditions and most importantly, thermostability. Growth rate of bacteria is much higher than fungal strains, which makes it easier for higher recombinant production of enzymes. Bacterial enzymes are also more complex in nature than their fungal counterparts, and hence provide improved function and synergy. Apart from this, the presence of bacterial strains in very harsh environmental conditions including extreme temperatures, pH, salt conditions, which often exist in the bioconversion chamber further adds an advantage. Fungal cellulases are extracted mainly from *Trichoderma* sp., *Penicillium* sp., *Fusarium* sp., *Phanerochaete* sp., *Humicola* sp., *Schizophyllum* sp., and *Aspergillus* sp. (Gupta and Verma, 2015; Agrawal et al., 2016; Srivastava et al., 2018). These species also produce enzymes which have high thermal stability (Maki et al., 2009). Cellulase production reported from *Paenibacillus* and *Bacillus* strains have stability at high temperature and wide pH range, while *Cellulomonas flavigena* and *Teredinibacter turnerae* produce enzymes with multifunctionality and a broader substrate utilization (Maki et al., 2009). *Pseudomonas campinasensis* BL11, a thermophilic bacterial isolate has growth temperatures over 25–60°C, wide pH range, and the ability to utilize a variety of saccharides and polysaccharides to produce xylanase, pectinase, cyclodextrin transferase, and two cellulases (Ko et al., 2007). Cellulosome production by *Clostridium thermocellum* and the ability to ferment sugars further offer prompts to use bacterial species for the bioconversion of biomass.

A novel thermophilic cellulose degrading bacterium (*Herbinix hemicellulosilytica* gen.) isolated from thermophilic biogas reactor (Koeck et al., 2015), and *Herbivorax saccincola* gen. nov., sp. nov., isolated by Koeck et al. (2016) from lab-scale biogas reactor, showed good total cellulase enzyme activities of 0.05 Umg⁻¹ on crystalline cellulose (Koeck et al., 2016), which is comparable to the activity shown by *C. thermocellum* which is 0.09 Umg⁻¹. Recent publications on thermophiles from Yellowstone national park suggest that thermophiles possess

good cellulolytic activity (Hamilton-Brehm et al., 2010). Another study by Najar et al. (2018), reported the isolation of highly thermophilic bacteria from a north-east Indian hot spring. Hence, there is a need to isolate new thermophilic bacterial species in order to boost the biofuel production industry.

The cellulolytic enzymes work efficiently at temperatures around 50°C and in some studies, even at higher temperatures (Baharuddin et al., 2016). Verma et al. (2018) isolated the cellulase producing thermophilic *Bacillus subtilis* BHUJP-H1, *Bacillus* sp. BHUJP-H2, *Bacillus licheniformis* BHUJP-H3 from hot spring of Leh and Ladakh, indicates that these samples would provide bacteria inhabiting extreme environment, high temperature, and pressure tolerance (high altitude). This gives the hope to screen the bacterial strains with high cellulose degrading ability and help in enhanced bioethanol production. In this study, consortia of cellulose degrading microbes was developed to increase the cellulase activity of the bacterial strains (Agrawal et al., 2018a,b). Wheat straw and sugarcane bagasse, two major agro-wastes produced in the Northern part of India, were used as substrates for cellulase production. The higher activity of the cellulases on these substrates can lead to higher production of glucose and enhance bioethanol production.

The utilization of waste biomass to produce ethanol through cellulose degrading microbes not only reduce the emissions produced from the wastes, but also reduce the greenhouse gas emissions from fossil fuels usage, when used in a blended form. Moreover, bioethanol is a promising clean renewable biofuel, used mainly as a fuel additive which enhances the engine performance and simultaneously reduces the air pollution. With conventional fuels, such as gasoline and petroleum evolving huge amounts of greenhouse gases into the atmosphere, biofuels become the need of the hour. The higher production of glucose and bioethanol from the cellulosic biomass can boost up the biofuel production industry and this bioethanol can be amended in the gasoline by 20% to help reduce the fossil fuel consumption (Gupta and Verma, 2015; Agrawal et al., 2018c). The main aim of this study is to isolate thermophilic bacterial strains for developing efficient cellulose degrading consortium for glucose production from different agro-residues.

MATERIALS AND METHODS

Isolation of Bacterial Strains

The water and soil samples were collected from the hot springs of Leh and Ladakh region of Jammu and Kashmir. The isolation was done through serial dilution and plating methods (Das et al., 2010). The plates were incubated at 45°C. The bacterial colonies were counted and picked the pure colonies on slant agar for further analysis. The pure cultures were also stored in 40% glycerol stock and kept at -80°C for further use. The bacterial strains were tested for the growth and morphology on nutrient agar plates at 48 h incubation. The strains were preliminarily tested for their cellulose degrading ability in carboxymethyl cellulose (CMC) plates. The plates were streaked with the bacterial strains on CMC agar media and incubated at 48–72 h for degradation of cellulose.

Abbreviations: CMC, Carboxymethyl Cellulose; NA, Nutrient agar; Uml⁻¹, International units per milliliter; ml, Milliliter; w/v, Weight/Volume; EDTA, Ethylene diamine tetra acetic acid; µg, Microgram; FPase, Enzyme activity on filter paper; mg, Milligram; °C, Degree Celsius; CO₂, Carbon Dioxide; N₂O, Dinitrogen oxide; CH₄, Methane; MR, Methyl Red; VP, Voges-Proskauer; OD, Optical density; nm, Nanometre; DNS, Dinitrosalicylic acid; h, Hours.

Biochemical Analysis

The selected strains were studied for their biochemical characterization. The biochemical tests performed were catalase test, Methyl Red Voges-Proskauer test, indole test, Gram's staining. Bacterial strains were grown in Mandel's medium and harvested at different intervals. CMC was used as the carbon source with 2% (w/v) mix in the Mandel's medium for determination of total cellulase and endoglucanase activity. Avicel (Sigmacell 20- Sigma Aldrich) with 2% (w/v) mix in the Mandel's media in place of CMC was used for the quantification of exoglucanase. For the estimation of β -glucosidase activity, p-nitrophenol- β -D-glucoside (p-NPG) with 400 μ g/ml was used as a carbon source in Mandel's medium (Strahsburger et al., 2017). Total cellulase and endoglucanase activities were calculated at 72 h and exoglucanase and β -glucosidase activities were calculated at 24, 48, and 72 h (Zhang et al., 2009).

Estimation of Total Cellulase Activity

Crude enzyme (500 μ l) was mixed with 50 mg of filter paper (Whatman No. 1) and 1.0 ml of 50 mM citrate buffer, then incubated for 1 h at 50°C. After that, 3 ml dinitro salicylic acid (DNS) reagent was added in reaction mixture to stop the reaction. The mixture was incubated in a water bath at 100°C for 10 min for the color development. The mixture was then allowed to cool and the optical density (OD) was measured at 540 nm (Miller et al., 1960). The OD of the enzyme assay was then used to calculate the concentration of the enzyme released against glucose standards. The FPase (Total cellulase) activity in U/ml was calculated using the following formula (Shareef et al., 2015):

$$FPase \frac{U}{ml} = \frac{\text{Concentration of Glucose in mg/ml}}{\text{Time of Incubation} \times \text{volume of substrate} \times \text{Molecular wt. of Glucose}} \times 1000$$

Estimation of Endoglucanase Activity

Carboxymethyl cellulose sodium salt (Hi-Media) (2% w/v) solution was prepared in citrate buffer. Five hundred microliters of the crude enzyme was mixed with 500 μ l of CMC (2% w/v) and incubated for 30 min at 50°C. After the incubation, the mixture was mixed with DNS reagent to arrest the reaction, then incubated in a water bath for 10 min at 100°C boiling water and later allowed to cool. Following that the OD of the mixture was taken at 540 nm. The CMCase activity in U/ml was calculated using following formula (Shareef et al., 2015):

$$CMCse \frac{U}{ml} = \frac{\text{Concentration of Glucose in mg/ml}}{\text{Time of Incubation} \times \text{volume of substrate} \times \text{Molecular wt. of Glucose}} \times 1000$$

Estimation of Exoglucanase Activity

Exoglucanase activity assay was performed using the protocol given by Zhang et al. (2009). Sigmacell 20 (Avicel) in the powder form (20 g) was mixed with 1,000 ml of acetate buffer. The bacterial cultures were grown in modified Mandel's medium with Avicel as a carbon source. Four hundred microliters of the crude enzymes were mixed with 1.6 ml of Avicel suspension (2% w/v). The mixtures were incubated for 2 h at 50°C. The reaction was stopped using 4 ml of glycine buffer. The hydrolysate produced

was then assayed with the help of phenol sulfuric acid assay (Zhang et al., 2009). The hydrolysate (0.7 ml) was mixed with 0.7 ml of 5% phenol and 3.5 ml of concentrated sulfuric acid for color development. The mixture was then allowed to cool and OD were taken at 490 nm.

$$Avicelase \frac{U}{ml} = \frac{\text{Concentration of Glucose in mg/ml}}{\text{Time of Incubation} \times \text{volume of substrate} \times \text{Molecular wt. of Glucose}} \times 1000$$

Estimation of β -Glucosidase Activity

p-Nitrophenol β -D-glucoside (5 mM) was used as substrate for the estimation of the β -glucosidase enzyme activity. The bacterial strains were grown in modified Mandel medium flooded with p-NPG as the carbon source. Crude enzyme (0.2 ml) was mixed with 1 ml of p-NPG suspension and 1.8 ml of acetate buffer. The mixtures were incubated at 50°C for 30 min. Four milliliters of glycine buffer was used to stop the reaction. The β -glucosidase activity in U/ml was calculated using this formula (Shareef et al., 2015):

$$\beta - \text{glucosidase} \frac{U}{ml} = \frac{\text{Concentration of p - Nitrophenol mg/ml}}{\text{Time of Incubation} \times \text{volume of substrate} \times \text{Molecular wt. of Glucose}} \times 1000$$

Using Sugarcane Bagasse and Wheat Straw for Estimation of Total Cellulase Enzyme in Term of Glucose Production

Sugarcane and wheat straw were used to estimate the effect of crude enzymes on the agro-residues. Sugarcane bagasse, collected from a nearby cane juice store (25.284708°N, 83.000852°E, variety: CO-238) was washed, and oven dried at 50°C for 2 days. It was crushed and then autoclaved before its final use for the estimation. The wheat straw brought from a nearby store (25.264407°N, 82.9950523°E, variety: Malviya 510) was oven dried, crushed and autoclaved before use (**Supplementary Figure 1**). For the estimation of the degradation of the substrate by the enzyme, the estimation of the individual enzyme activity was measured (Zhang et al., 2009). The quantity of the substrates taken were 50 mg sugarcane bagasse and 50 mg wheat straw. The enzyme activity on sugarcane and wheat straw is calculated in U/ml by the following formula:

$$\begin{aligned} \text{Enzyme activity on sugarcane bagasse} & \frac{mg}{ml} \\ & = \text{Concentration of Glucose in} \frac{mg}{ml} \\ \text{Enzyme activity on wheat straw} & \frac{mg}{ml} \\ & = \text{Concentration of Glucose in} \frac{mg}{ml} \end{aligned}$$

Total Cellulase, Endoglucanase, Exoglucanase and β -Glucosidase Activities Estimated in Different Combinations of Three Bacterial Strains

Total cellulase, endoglucanase, exoglucanase, and β -glucosidase enzyme activities were checked in presence of three consortia

prepared from three bacterial strains (*B. subtilis* BHUJPV-H19, *Bacillus stratosphericus* BHUJPV-H5, and *B. subtilis* BHUJPV-H12). Initially the cultures were grown in nutrient broth for overnight (12 h). Consortia were prepared by inoculating equal volume of broth cultures into the Mandel's media with respective carbon sources for different enzyme activities to be quantified. The CMC was the carbon source used in Mandel's medium for total cellulase and endoglucanase activity estimation while avicel and p-nitrophenol- β -D-glucoside were used as carbon sources for the estimation of exoglucanase and β -glucosidase activities, respectively, by modified method of Zhang et al. (2009).

16S rDNA Gene Amplification

The molecular identification of the bacterial strains was done through 16S rDNA sequencing. The isolation of genomic DNA was done by modified method of Russell and Sambrook (2001).

Amplification of the 16S rDNA

The universal primers were used for the amplification of 16S rDNA gene of each bacterial strains. The forward primer 27F (50-AGAGTTTGATCCTGGCTCAG-30) and reverse primers 939R (5'-CTTGTGCGGGCCCCCGTCAATTC-3') and 1492R (5'-CAGGAAACAGCTATGAC-3') were used [14]. The amplified product was purified using PCR purification kit (Invitrogen, PureLink™ PCR purification kit, United States) for the sequencing of 16S rDNA gene. BLAST (basic local alignment sequence tool) was done of partial 16S rDNA gene sequence from NCBI data base. The obtained results with high similarity were used for identification of bacterial strains on the basis of query cover and identity percentage. Then partial 16S rDNA gene sequence submitted in the NCBI for getting accession number. The results of the 16S rDNA sequencing were used to establish the genetic relationship between the isolated bacterial strains. The software used for the purpose was Clustal W and Mega 4.0 with 500 bootstrap replications.

Statistical Analysis

All the enzymatic activities were carried out in triplicate and the results were expressed as mean \pm standard error of independent replicates. Analysis of variance (ANOVA) with Duncan *post hoc* test was performed using SPSS (Statistical Package for the Social Sciences) software (version 21). The values of $P \leq 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Morphology and Biochemical Characterization of the Bacterial Strains

The isolated microbial strains showed different types of colony forms such as irregular flat type colony form with entire margins, punctiform, filamentous, and circular types. Also, strains such *B. subtilis* BHUJPV-H14 and *B. subtilis* BHUJPV-H22 showed raised elevation and *B. subtilis* BHUJPV-H23 and *B. altitudinis* BHUJPV-H7 showed convex elevation. The margins in the colony form of the bacterial cultures were mostly entire but some of them showed curled and undulate forms such as *B. subtilis*

BHUJPV-H14, *B. subtilis* BHUJPV-H23, and *B. subtilis* BHUJPV-H19 with curled margins and *B. altitudinis* BHUJPV-H7 with undulate margins (**Supplementary Figure 2; Table 1**).

All strains showed positive test for Gram's staining and found rod shape cells. All strains showed negative results for Indole test. *B. subtilis* BHUJPV-H14 and *B. subtilis* BHUJPV-H19 showed negative results for catalase test while all others showed positive results for catalase test. In methyl-red test the *B. subtilis* BHUJPV-H14, *B. frigiditolerans* BHUJPV-H24, *B. frigiditolerans* BHUJPV-H6, and *B. aryabhatai* BHUJPV-H26 were found positive while others showed negative results for the same. The Voges-Proskauer test showed positive results in *B. subtilis* BHUJPV-H14, *B. subtilis* BHUJPV-H23, *B. frigiditolerans* BHUJPV-H6, and *B. aryabhatai* BHUJPV-H26, and the remaining others showed negative results for the test. All the strains showed positive results for cellulase test qualitatively with good zone of clearance and hence were taken further for the quantification of the enzyme activities, along with their molecular identification (**Supplementary Figures 3, 4; Table 2**).

Molecular Identification of Bacteria Strains

16S rDNA gene sequencing was done and submitted in NCBI followed by BLAST result. The sequence for these strains were submitted in NCBI to obtain the accession number. Most of the identified bacterial strains were showed maximum similarity with *Bacillus* strains with BLAST results. After BLAST result, the bacterial strains were identified as *B. subtilis* BHUJPV-H14 (Accession number: MG550890), *B. subtilis* BHUJPV-H25 (Accession number: MG596982), *B. subtilis* BHUJPV-H22 (Accession number: MG596980), *B. frigiditolerans* BHUJPV-H24 (Accession number: MG596981), *B. subtilis* BHUJPV-H23 (Accession number: MG596987), *B. subtilis* BHUJPV-H19 (Accession number: MG596986), *B. frigiditolerans* BHUJPV-H6 (Accession number: MF664678), *B. aryabhatai* BHUJPV-H26 (Accession number: MG596983), *B. stratosphericus* BHUJPV-H5 (Accession number: MF664677), *B. subtilis* BHUJPV-H12 (Accession number: MF776630), and *B. altitudinis* BHUJPV-H7 (Accession number: MF664679). Many *Bacillus* strains have been reported in the past that have been isolated from samples of high temperatures. Yadav et al. (2015), reported nine genera

TABLE 1 | External morphology of the selected bacterial strains.

Bacterial strains	Colony form	Elevation	Margin	Agar stroke (form of growth)
BHUJPV-H14	Irregular	Raised	Curled	Effuse
BHUJPV-H25	Irregular	Flat	Entire	Beaded
BHUJPV-H22	Irregular	Raised	Entire	Filliform
BHUJPV-H24	Punctiform	Flat	Entire	Beaded
BHUJPV-H23	Filamentous	Convex	Curled	Beaded
BHUJPV-H19	Circular	Flat	Curled	Beaded
BHUJPV-H6	Irregular	Flat	Entire	Filliform
BHUJPV-H26	Irregular	Flat	Entire	Beaded
BHUJPV-H5	Irregular	Flat	Entire	Filliform
BHUJPV-H12	Irregular	Flat	Entire	Effuse
BHUJPV-H7	Punctiform	Convex	Undulate	Effuse

TABLE 2 | Biochemical characterization of the selected bacterial strains.

Bacterial strains	Gram's staining	Shape of bacteria	Indole test	Catalase test	Methyl red	Voges-proskauer	Fluorescence test	Cellulase
<i>Bacillus subtilis</i> BHUJVP-H14	Positive	Bacillus	Negative	Negative	Positive	Positive	Negative	Positive
<i>Bacillus subtilis</i> BHUJVP-H25	Positive	Bacillus	Negative	Positive	Negative	Negative	Negative	Positive
<i>Bacillus subtilis</i> BHUJVP-H22	Positive	Bacillus	Negative	Positive	Negative	Negative	Negative	Positive
<i>Brevibacterium frigoritolerans</i> BHUJVP-H24	Positive	Bacillus	Negative	Positive	Positive	Negative	Negative	Positive
<i>Bacillus subtilis</i> BHUJVP-H23	Positive	Bacillus	Negative	Positive	Negative	Positive	Negative	Positive
<i>Bacillus subtilis</i> BHUJVP-H19	Positive	Bacillus	Negative	Negative	Negative	Negative	Negative	Positive
<i>Brevibacterium frigoritolerans</i> BHUJVP-H6	Positive	Bacillus	Negative	Positive	Positive	Positive	Negative	Positive
<i>Bacillus aryabhatai</i> BHUJVP-H26	Positive	Bacillus	Negative	Positive	Positive	Positive	Negative	Positive
<i>Bacillus stratosphericus</i> BHUJVP-H5	Positive	Bacillus	Negative	Positive	Negative	Negative	Negative	Positive
<i>Bacillus subtilis</i> BHUJVP-H12	Positive	Bacillus	Negative	Positive	Negative	Negative	Negative	Positive
<i>Bacillus altitudinis</i> BHUJVP-H7	Positive	Bacillus	Negative	Positive	Negative	Negative	Negative	Positive

that belong to Bacillaceae which are, *Bacillus*, *Halobacillus*, *Lysinibacillus*, *Oceanobacillus*, *Paenibacillus*, *Salinibacillus*, *Sediminibacillus*, *Thallasobacillus*, and *Virgibacillus*. Another study by Acharya and Chaudhary (2012), reported the isolation of *B. licheniformis* WBS1 and *Bacillus* sp. WBS from a hot spring West Bengal. Mohammad et al. (2017), identified isolated *B. licheniformis* and *Thermomonas hydrothermalis* from Jordan hot springs.

In the phylogenetic tree analysis, bacterial isolates *B. altitudinis* BHUJVP-H14 and BHUJVP-H19 were closely related. Strains *B. frigoritolerans* BHUJVP-H24 and *B. aryabhatai* BHUJVP-H26 to *B. stratosphericus* BHUJVP-H5 to *B. altitudinis* BHUJVP-H7 showed very close similarity in the phylogenetic tree analysis. *B. subtilis* BHUJVP-H22 to *B. subtilis* BHUJVP-H25, *B. subtilis* BHUJVP-H14 to *B. subtilis* BHUJVP-H19 were found to be very closely related. *B. aryabhatai* BHUJVP-H26 and *B. frigoritolerans* IND-13 showed close similarity. These two strains are also closely related to *B. altitudinis* HEM05. *B. stratosphericus* BHUJVP-H5 is also closely related to *B. subtilis* KRI, *B. subtilis* BHUJVP-H14 and *B. subtilis* BHUJVP-H19. *B. frigoritolerans* BHUJVP-H6 is closely related to *B. subtilis* BHUJVP-H12, *B. aryabhatai* CN13-5, *B. subtilis* GX S18, *B. subtilis* GX S-5 (Supplementary Figure 5).

Enzyme Quantification

Estimation of Total Cellulase Activity in Bacterial Strains

The total cellulase activity of the identified bacterial strains ranged as high as 0.14 Uml⁻¹. Bacterial strains *B. stratosphericus* BHUJVP-H5, *B. subtilis* BHUJVP-H12, *B. subtilis* BHUJVP-H19, and *B. subtilis* BHUJVP-H23 showed 0.14, 0.13, 0.12, and 0.14 Uml⁻¹ total cellulase enzyme activity, respectively (Figure 1). Bacterial strains such as *B. subtilis* BHUJVP-H14 and *B. frigoritolerans* BHUJVP-H24 showed total cellulase activities of 0.03 and 0.04 Uml⁻¹. All other bacterial strains showed low total cellulase activity when quantified. In a similar study, by Dipasquale et al. (2014), maximum cellulase activity was found to be 10.3 Uml⁻¹ (purified crude extract) in *Thermosipho* sp. strain

3. In a study by Al Azkawi et al. (2018) *B. subtilis* S1 was found to be producing 2.963 U/ml of cellulase enzyme.

Estimation of Endoglucanase Activity in Bacterial Strains

Similarly, high endoglucanase enzyme activities were observed in *B. stratosphericus* BHUJVP-H5, *B. subtilis* BHUJVP-H12, *B. subtilis* BHUJVP-H19, and *B. subtilis* BHUJVP-H23 with activities 0.35, 0.21, 0.23, and 0.26 Uml⁻¹, respectively, at 72 h. Other strains such as *B. subtilis* BHUJVP-H22 showed moderate amount of endoglucanase activity (0.08 Uml⁻¹). All other bacterial strains produced enzyme at a much lower activity rate with 0.03 Uml⁻¹ by *B. subtilis* BHUJVP-H14, 0.02 Uml⁻¹ by *B. subtilis* BHUJVP-H25, *B. frigoritolerans* BHUJVP-H24, *B. frigoritolerans* BHUJVP-H6, and *B. aryabhatai* BHUJVP-H26 while 0.04 Uml⁻¹ endoglucanase enzyme activity was observed in *B. altitudinis* BHUJVP-H7 at 72 h incubation (Figure 2). Similarly, Ladeira et al. (2015) observed the endoglucanase activity 0.29 Uml⁻¹ by *Bacillus* sp. at 50°C incubation.

Estimation of Exoglucanase Activity in Bacterial Strains

The exoglucanase activity of the bacterial strains ranged as high as 0.06 Uml⁻¹ at 24 h of enzyme harvest and its assay in *B. aryabhatai* BHUJVP-H26. The *B. altitudinis* BHUJVP-H7 showed enzyme activity of 0.03 Uml⁻¹, while *B. subtilis* BHUJVP-H22, *B. subtilis* BHUJVP-H23, *B. frigoritolerans* BHUJVP-H6, and *B. stratosphericus* BHUJVP-H5 showing exoglucanase activity of 0.02 Uml⁻¹ at 24 h incubation (Figure 3). At 48 h incubation, no efficient exoglucanase activity was observed in the bacterial cultures except *B. subtilis* BHUJVP-H12 which showed an activity of 0.03 Uml⁻¹. Other strains such as *B. subtilis* BHUJVP-H14, *B. frigoritolerans* BHUJVP-H6, *B. stratosphericus* BHUJVP-H5, and *B. altitudinis* BHUJVP-H7 showed an activity of 0.02 Uml⁻¹. Remaining strains showed very low exoglucanase activity at 48 h of enzyme harvest. The exoglucanase activity of the bacterial strains with enzymes harvested at 72 h showed

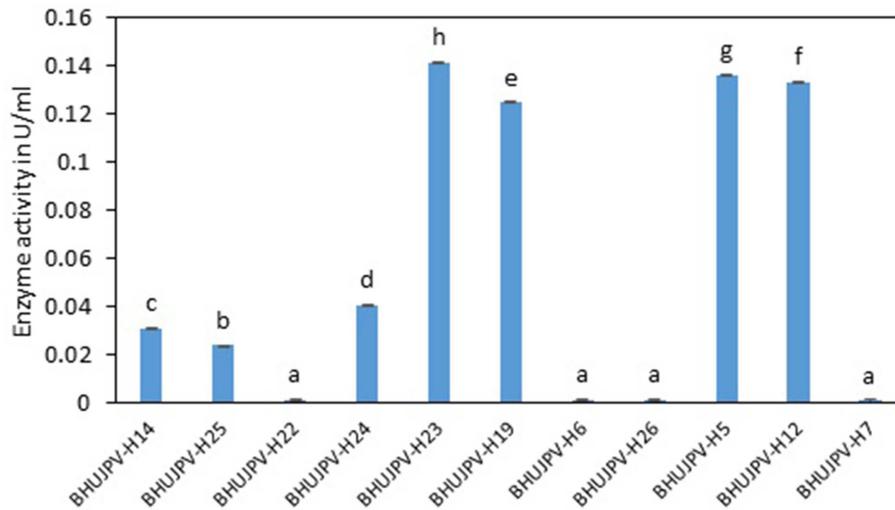


FIGURE 1 | Total cellulase activity of the isolated bacterial strains at 72 h of enzyme harvest. Values are the mean \pm SE (standard error). Mean values in each bar with the same alphabet superscript(s) do not differ significantly, but different alphabet superscript is showed significantly different between each treatment by Duncan *post hoc* test ($p \leq 0.05$).

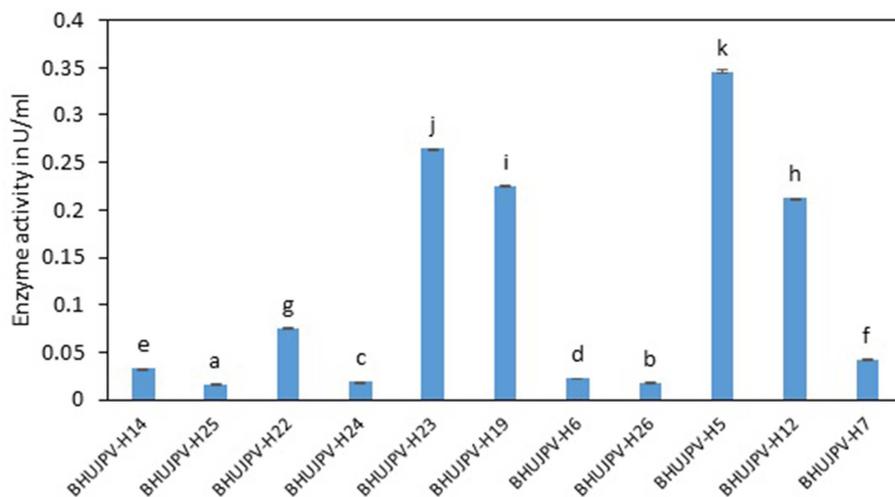


FIGURE 2 | Endoglucanase activity of the isolated bacterial strains at 72 h of enzyme harvest. Values are the mean \pm SE (standard error). Mean values in each bar with the same alphabet superscript(s) do not differ significantly, but different alphabet superscript is showed significantly different between each treatment by Duncan *post hoc* test ($p \leq 0.05$).

somewhat similar results to 24 and 48 h enzyme harvest. The exoglucanase activity of the isolated bacterial strains at 72 h of enzyme harvest ranged upto 0.02 Uml^{-1} . *B. subtilis* BHUJVPV-H14 and *B. subtilis* BHUJVPV-H12 showed 0.02 Uml^{-1} of enzyme activity while other strains such as *B. subtilis* BHUJVPV-H25, *B. subtilis* BHUJVPV-H22, *B. frigiditolerans* BHUJVPV-H24, *B. subtilis* BHUJVPV-H19, *B. frigiditolerans* BHUJVPV-H6, *B. aryabhatai* BHUJVPV-H26, *B. altitudinis* BHUJVPV-H7 showed enzyme activities of 0.01 Uml^{-1} . Ladeira et al. (2015) also studied the exoglucanase activity in *Bacillus* sp. and found it to be 0.83 Uml^{-1} .

β -Glucosidase Activity

The β -glucosidase activity of the isolated bacterial cultures at 24 h of harvest ranged as high as 5.03 Uml^{-1} in *B. altitudinis* BHUJVPV-H7. *B. subtilis* BHUJVPV-H19, *B. frigiditolerans* BHUJVPV-H6, and *B. subtilis* BHUJVPV-H12 showed enzyme activity of 2.55 , 2.63 , and 1.24 Uml^{-1} , respectively. Other strains such as *B. subtilis* BHUJVPV-H25, *B. subtilis* BHUJVPV-H23, and *B. stratosphericus* BHUJVPV-H5 showed enzyme activities of 1.30 , 1.87 , and 1.33 Uml^{-1} , respectively. *B. subtilis* BHUJVPV-H22, *B. frigiditolerans* BHUJVPV-H24, and *B. aryabhatai* BHUJVPV-H26 showed activities of 0.43 , 0.50 , and 0.40 Uml^{-1} . The enzyme

activities harvested at 48 h from *B. subtilis* BHUJPV-H23 was 4.14 Uml^{-1} . *B. subtilis* BHUJPV-H25, *B. stratosphericus* BHUJPV-H5, and *B. subtilis* BHUJPV-H12 showed enzyme activities of 3.53, 1.37, and 0.43 Uml^{-1} , respectively. The other strains such as *B. subtilis* BHUJPV-H14, *B. subtilis* BHUJPV-H22, *B. frigiditolerans* BHUJPV-H24, *B. subtilis* BHUJPV-H19, *B. frigiditolerans* BHUJPV-H6, *B. aryabhatai* BHUJPV-H26, and *B. altitudinis* BHUJPV-H7 showed enzyme activities of 0.79, 0.36, 0.83, 0.54, 0.22, 0.65, and 0.79 Uml^{-1} , respectively, at 48 h of enzyme harvest from the bacterial cultures. The enzyme activities of the isolated bacterial strains at 72 h of incubation ranged as high as 5.65 Uml^{-1} in *B. subtilis* BHUJPV-H19. The enzyme activities of other bacterial strains at 72 h of incubation include *B. subtilis* BHUJPV-H23, and *B. aryabhatai* BHUJPV-H26 with enzyme activities of 4.21, and 4.14 Uml^{-1} . *B. subtilis* BHUJPV-H14, *B. subtilis* BHUJPV-H22, *B. frigiditolerans*

BHUJPV-H24, *B. frigiditolerans* BHUJPV-H6, *B. stratosphericus* BHUJPV-H5, and *B. altitudinis* BHUJPV-H7 were found to possess enzyme activities of 0.43, 0.43, 0.54, 0.86, 0.50, and 0.43 Uml^{-1} , respectively, at 72 h incubation (Figure 4). Sharma et al. (2015) observed β -glucosidase activity of 3.1 Uml^{-1} by *P. janthinellum* EMS-UV-8. High β -glucosidase activity can improve the overall rate of reaction by reducing end product inhibition (Agrawal et al., 2016, 2017).

Effect of Cellulase Enzyme Activity on Different Agro-Residues

Enzyme Cellulase Activity on Sugarcane Bagasse

A very high activity was shown by *B. subtilis* BHUJPV-H19 with 6.06 mg/ml of glucose released in the hydrolysate with 60 min exposure to the sugarcane bagasse. *B. subtilis* BHUJPV-H23

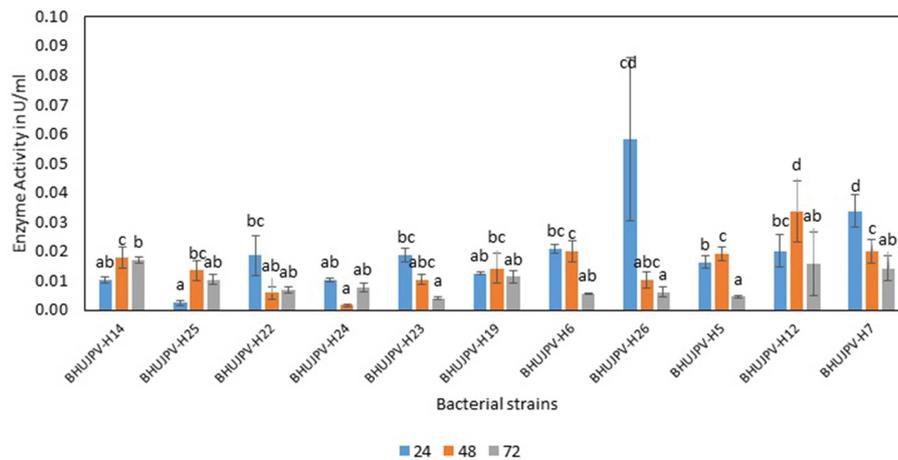


FIGURE 3 | Exoglucanase enzyme activity of the bacterial strains in the modified Mandel media at different time intervals. Values are the mean \pm SE (standard error). Mean values in each bar with the same alphabet superscript(s) do not differ significantly, but different alphabet superscript is showed significantly different between each treatment by Duncan *post hoc* test ($p \leq 0.05$).

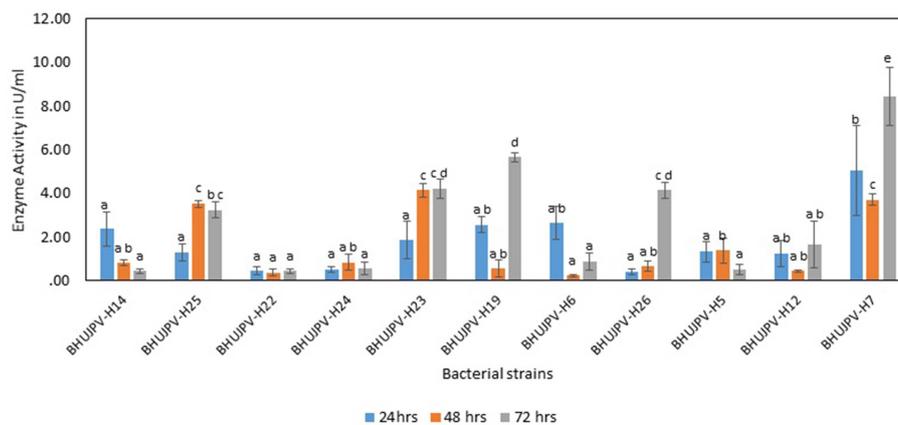


FIGURE 4 | β -glucosidase activity of different bacterial strains. Values are the mean \pm SE (standard error). Mean values in each bar with the same alphabet superscript(s) do not differ significantly, but different alphabet superscript is showed significantly different between each treatment by Duncan *post hoc* test ($p \leq 0.05$).

released 4.69 mg/ml of glucose. Similarly, *B. stratosphericus* BHUJVPV-H5 and *B. subtilis* BHUJVPV-H12 allowing the release of 4.62 and 4.42 mg/ml of glucose in the hydrolysate. The strains *B. subtilis* BHUJVPV-H14, *B. subtilis* BHUJVPV-H25, *B. subtilis* BHUJVPV-H22, *B. fragoriterans* BHUJVPV-H24, BHUJVPV-H26, and *B. altitudinis* BHUJVPV-H7 released low amounts of glucose in the hydrolysate, with 0.10, 0.02, 0.11, 0.10, 0.30, 0.11, and 0.14 mg/ml of glucose, respectively, at 72 h of enzyme harvest (Figure 5).

Enzyme Cellulase Activity on Wheat Straw

Comparatively lower activity was shown on wheat straw than the sugarcane bagasse by the isolated cellulolytic bacterial strains. The strains *B. subtilis* BHUJVPV-H12, *B. subtilis* BHUJVPV-H23,

B. subtilis BHUJVPV-H19, *B. fragoriterans* BHUJVPV-H6, and *B. altitudinis* BHUJVPV-H7 showed higher glucose release of 0.72, 0.48, 0.39, 0.22, and 0.24 mg/ml, respectively. Other strains *B. subtilis* BHUJVPV-H14, *B. subtilis* BHUJVPV-H25, *B. subtilis* BHUJVPV-H22, *B. fragoriterans* BHUJVPV-H24, and *B. aryabhattai* BHUJVPV-H26 released 0.03, 0.23, 0.22, 0.03, and 0.04 mg/ml of glucose, respectively, at 72 h of enzyme harvest (Figure 6).

A study on raw poplar biomass by Tabassum et al. (2018) observed the release of 2.30 mg/ml of reducing sugars after 6 h of incubation, while the commercial cellulase released 3.85 mg/ml reducing sugars. The bacterial strains in the form of consortia in general showed the increase in the enzyme activity in comparison to the enzyme harvest of a single bacterial

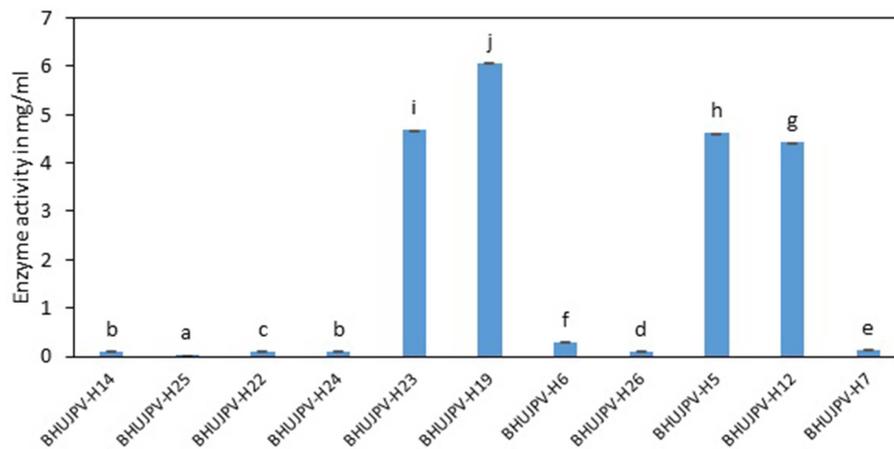


FIGURE 5 | Enzyme activity on wheat straw (Sugars released from sugarcane bagasse). Values are the mean \pm SE (standard error). Mean values in each bar with the same alphabet superscript(s) do not differ significantly, but different alphabet superscript is showed significantly different between each treatment by Duncan *post hoc* test ($p \leq 0.05$).

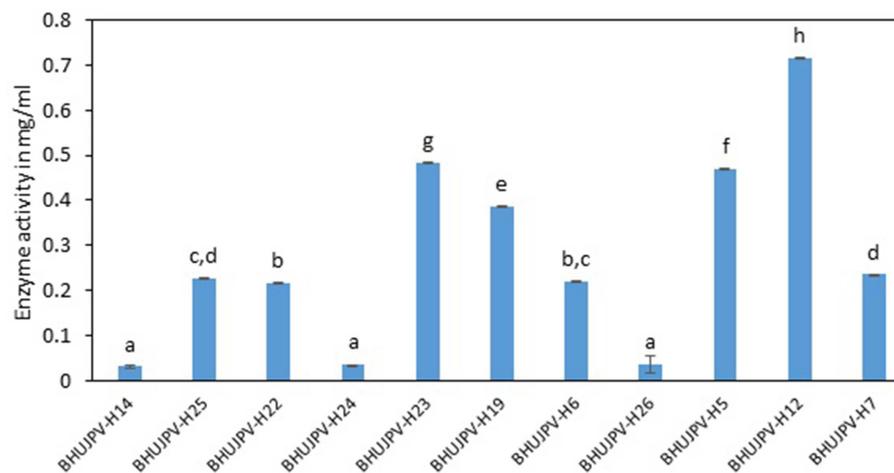


FIGURE 6 | Enzyme activity on sugarcane bagasse (Sugars released on degrading wheat straw). Values are the mean \pm SE (standard error). Mean values in each bar with the same alphabet superscript(s) do not differ significantly, but different alphabet superscript is showed significantly different between each treatment by Duncan *post hoc* test ($p \leq 0.05$).

strain. The enzyme activities of all four enzymes were quantified at different time intervals, i.e., 24, 48, and 72 h. Kato et al. (2005), studied the coexistence of five bacterial isolates namely *Clostridium straminisolvans* CSK1, *Clostridium* sp. strain FG4, *Pseudoxanthomonas* sp. strain M1-3, *Brevibacillus* sp. strain M1-5, and *Bordetella* sp. strain M1-6, in the form of mixed cultures to study the coexistence among themselves, which exhibited functional and structural stability. Here in this study, we have focused on the higher enzyme cultivation study, rather than going for the mechanism involved behind the same. A study by Nair et al. (2018) successfully optimized the cellulase activity to 2.42 U/ml in *B. velezensis* ASN1. A similar kind of study was done by Sivakumar et al. (2016) to isolate and characterize *B. licheniformis* from compost.

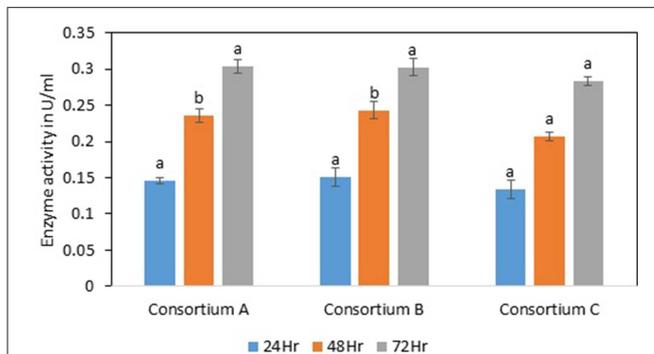


FIGURE 7 | Total cellulase activity of different consortia. Values are the mean \pm SE (standard error). Mean values in each bar with the same alphabet superscript(s) do not differ significantly, but different alphabet superscript is showed significantly different between each treatment by Duncan *post hoc* test ($p \leq 0.05$).

Consortium A

The total cellulase activity was found to be high in consortium A with 0.15 Uml⁻¹ at 24 h, 0.24 Uml⁻¹ at 48 h, and 0.30 Uml⁻¹ at 72 h of enzyme harvest. When compared with the total cellulase activities of the individual bacterial strains, it was found that the total cellulase activity at 72 h was on lower side. The endoglucanase activity of the consortium exhibited 0.02, and 0.21 Uml⁻¹ of enzyme activity at 24, and 72 h of incubation and subsequent enzyme harvest, respectively. Endoglucanase activity of the isolated bacterial strains did not show higher enzyme activity in the form of consortium than the individual bacterial strains. The β -glucosidase activity of prepared Consortium A showed 8.15 Uml⁻¹ at 72 h of enzyme harvest (Figures 7–10).

Consortium B

The total cellulase activity of consortium B was also higher than the individual strains. The total enzyme activity of consortium B was 0.15, 0.24, and 0.30 Uml⁻¹ at 24, 48, and 72 h of enzyme harvest, respectively. The endoglucanase activity in consortium B also showed similar results, with lower endoglucanase activity, 0.05, 0.05, and 0.13 Uml⁻¹ of enzyme activity at 24, 48, and 72 h of enzyme cultivation, respectively, when compared with enzyme activity of individual strains with an activity of either 0.23 Uml⁻¹ or 0.21 Uml⁻¹. The exoglucanase activities of the prepared consortium was found to be 0.01, 0.00, and 0.00 Uml⁻¹ at 24, 48, and 72 h of enzyme harvest, respectively, which is again almost negligible (Figures 7–10). The β -glucosidase activity of the prepared Consortium B showed some good results. Enzyme activity was found to be 3.89, 6.16, and 2.91 Uml⁻¹ at 24, 48, and 72 h of enzyme harvest, respectively.

Consortium C

The total cellulase activity of the prepared consortia yet again showed good results in consortium C. Total cellulase activity

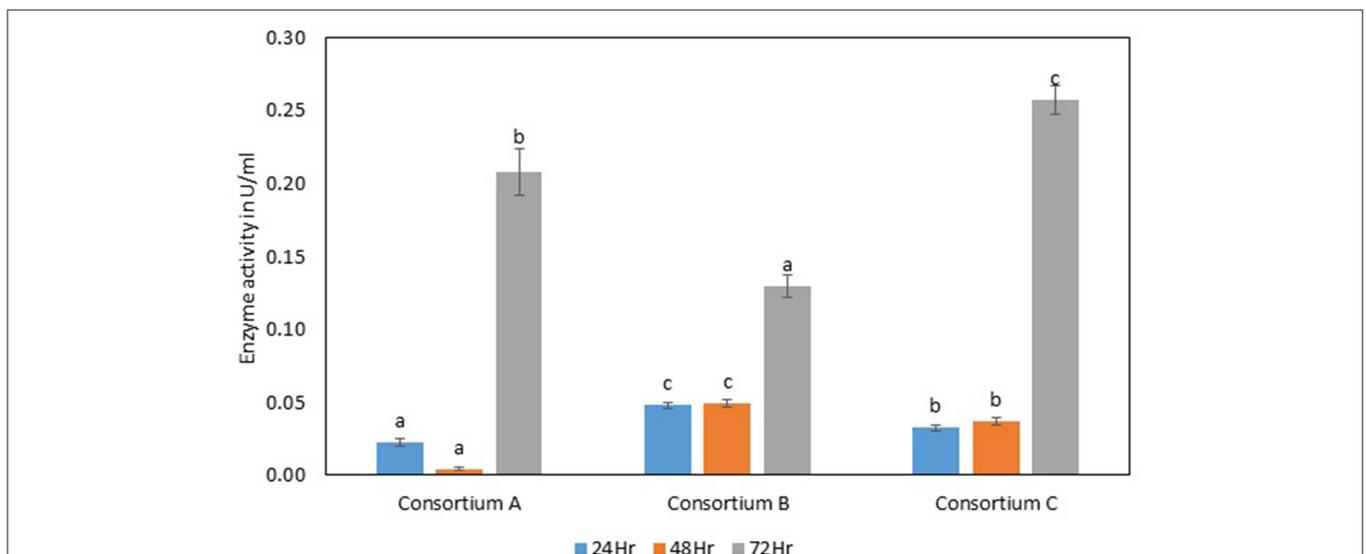


FIGURE 8 | Endoglucanase activity of different consortia. Values are the mean \pm SE (standard error). Mean values in each bar with the same alphabet superscript(s) do not differ significantly, but different alphabet superscript is showed significantly different between each treatment by Duncan *post hoc* test ($p \leq 0.05$).

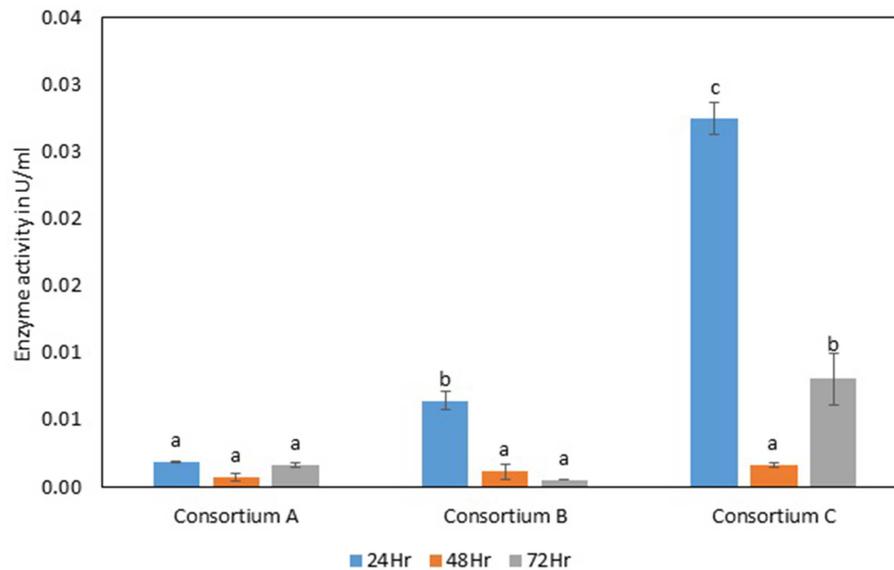


FIGURE 9 | Exoglucanase activity of different strains. Values are the mean \pm SE (standard error). Mean values in each bar with the same alphabet superscript(s) do not differ significantly, but different alphabet superscript is showed significantly different between each treatment by Duncan *post hoc* test ($p \leq 0.05$).

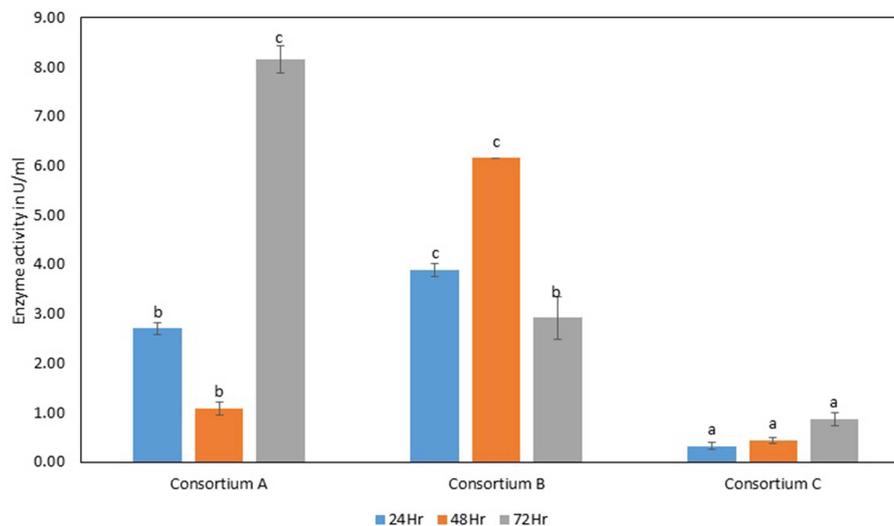


FIGURE 10 | beta-glucosidase activity of different strains. Values are the mean \pm SE (standard error). Mean values in each bar with the same alphabet superscript(s) do not differ significantly, but different alphabet superscript is showed significantly different between each treatment by Duncan *post hoc* test ($p \leq 0.05$).

by consortium C showed 0.13, 0.21, and 0.28 Uml^{-1} at 24, 48, and 72 h, respectively, which was higher in comparison to individual total cellulase activities of the bacterial strains. The endoglucanase activity of the consortium C showed low activity in comparison to individual bacterial strains with 0.03, 0.04, and 0.26 Uml^{-1} at 24, 48, and 72 h, respectively. Exoglucanase activity in the consortium C was also found to be on lower side in comparison to enzyme activity of individual bacterial strains. The activity was found to be 0.02, 0.00, and 0.01 Uml^{-1} at 24, 48, and 72 h, respectively. beta-glucosidase activity of consortium C

was found to be 0.32, 0.43, and 0.86 Uml^{-1} at 24, 48, and 72 h, respectively (Figures 7–10). This enzyme activity in consortium C was found to be very low in comparison to enzyme activity of individual bacterial strains.

In a study by Dabhi et al. (2014), it was observed that a highest of 0.178 Uml^{-1} of total cellulase activity, 1.716 Uml^{-1} of endoglucanase activity, and 0.602 Uml^{-1} of beta-glucosidase activity through the bacterial isolates in the form of consortia. Another study by Park et al. (2012), observed the release of 0.466 Uml^{-1} of endoglucanase, 0.014 Uml^{-1} of exoglucanase, and

TABLE 3 | A comparative study of enzyme activities of different microbes in literature and present study.

Name of microbe	Total cellulase activity	Endoglucanase activity	Exoglucanase (Avicelase) activity	β -glucosidase activity	References
<i>Bacillus</i> sp.	–	0.29 Uml ⁻¹	0.83 Uml ⁻¹	–	Ladeira et al., 2015
<i>Bacillus pumilus</i> EB3	0.011 Uml ⁻¹	0.079 Uml ⁻¹	–	0.038 Uml ⁻¹	Ariffin et al., 2006
Actinomycete	0.734 Uml ⁻¹	1.38 Uml ⁻¹	–	–	Mohanta, 2014
<i>Streptomyces</i> DSK59	27 Uml ⁻¹	–	–	–	Budihal et al., 2016
<i>B. velezensis</i> ASN1	2.42 Uml ⁻¹	–	–	–	Nair et al., 2018
<i>B. subtilis</i> S1	2.963 Uml ⁻¹	–	–	–	Al Azkawi et al., 2018
<i>Thermosipho</i> sp. strain 3	10.3 Uml ⁻¹	–	–	–	Dipasquale et al., 2014
<i>P.janthinellum</i> EMS-UV-8.	1.8 Uml ⁻¹	–	–	3.1 Uml ⁻¹	Sharma et al., 2015
<i>Bacillus stratosphericus</i> BHUJPV-H5	0.14 Uml ⁻¹	0.35 Uml ⁻¹	0.02 Uml ⁻¹	1.37 Uml ⁻¹	This study
<i>Bacillus subtilis</i> BHUJPV-H12	0.13 Uml ⁻¹	0.21 Uml ⁻¹	0.03 Uml ⁻¹	1.66 Uml ⁻¹	This study
<i>Bacillus subtilis</i> BHUJPV-H19	0.12 Uml ⁻¹	0.23 Uml ⁻¹	0.01 Uml ⁻¹	5.65 Uml ⁻¹	This study
Consortia A (BHUJPV-H5+BHUJPV-H19)	0.30 Uml ⁻¹	0.21 Uml ⁻¹	0.01 Uml ⁻¹	8.15 Uml ⁻¹	This study

0.041 Uml⁻¹ of β -glucosidase, when the cultures were used in the form of consortia. The enzyme activities in the consortia showed two-fold increase in total cellulase activity, but the endoglucanase activity decreased significantly. The results obtained in the quantification of the total cellulase enzyme revealed that the total production was much higher than other *B. subtilis* strains or the work carried out in the literature. The total cellulase activity in a similar work done Gupta et al. (2012), yielded 0.012–0.196 U/ml. It was reported that *B. subtilis* showed 1.08 U/mg total cellulase activity (Heck et al., 2002). In a recent study by Ladeira et al. (2015), the avicelase or exoglucanase activity was estimated to be 0.83 Uml⁻¹ and endoglucanase activity 0.29 Uml⁻¹ at cultivation time of 120 and 168 h, respectively. A study by Dipasquale et al. (2014) was carried out on a thermophilic microbe showing cellulose degradation as well as detergent compatibility. Another study with different lignocellulolytic enzyme activity in the consortium yielded results of CMCase 3.8 Umg⁻¹, xylanase 8.9 Umg⁻¹ at pH 5 on day two, and β -glucosidase activity of 5.0 Umg⁻¹ on day five. A comparative study of different microbes isolated and their maximum enzyme activities is mentioned in the **Table 3**. In this study, better results were found in terms of enzyme activity and might prove beneficial to the bio-based product industries. Further investigation of the consortia on different agro-residues can yield fruitfully significant results.

Future Perspectives

The thermophilic bacterial strains can be effectively used for the consolidated bioprocessing approach and also favors the rate of reaction in terms of hydrolysis product. These

strains can also be tested for their activity on different agro-residues such as rice husk, cotton dust. The thermophiles can also be used effectively if the substrate is pre-treated with the help of ionic liquids and eutectic solvents (Satlewal et al., 2018a,b). Ionic liquids are known to effectively loosen up the lignin-hemicellulose-cellulose complex (Satlewal et al., 2018b). Another major insight that can be looked into is the production of ligninolytic enzymes and other hemicellulose degrading enzymes by the identified bacterial strains. This may help in combined biomass processing of the agro-wastes. The bioprocessing approach together with the use of thermophiles could lead to the production of highly cost-effective bioethanol.

CONCLUSIONS

The bacterial strains isolated from hot water spring samples have been identified as mostly *Bacillus* species and some *Brevibacterium* species. Bacterial strain *B. stratosphericus* BHUJPV-H5 showed endoglucanase activity of 0.35 Uml⁻¹ at 72 h of enzyme harvest. *B. subtilis* BHUJPV-H12 showed β -glucosidase enzyme activity of 3.78 Uml⁻¹ with high sugar release of 4.42 and 0.72 mgml⁻¹ in sugarcane bagasse and wheat straw, respectively. *B. subtilis* BHUJPV-H19 showed β -glucosidase enzyme activity of 5.65 Uml⁻¹. Consortium A showed total cellulase enzyme activity of 0.30 Uml⁻¹, endoglucanase activity of 0.21 Uml⁻¹, and high β -glucosidase activity of 8.15 Uml⁻¹ at 72 h. Hence, this consortium A can be used for further modifications and also higher production of glucose from the agro-residues.

DATA AVAILABILITY

The datasets for this manuscript are not publicly available because we have submitted this research manuscript as original and novel research for making microbial consortia for cellulose degradation. All data is original not published anywhere, only molecular characterized data 16SrRNA sequences have been submitted in NCBI-DATA for getting accession numbers. Requests to access the datasets should be directed to JV, verma_bhu@yahoo.co.in.

AUTHOR CONTRIBUTIONS

This research was conducted and written by SS. Experiment designed and edited by JV and NS. DJ as co-author did isolation and characterization of all microbial strains which has been used in this study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fenrg.2019.00061/full#supplementary-material>

Supplementary Figure 1 | Pre-treated wheat straw and sugarcane bagasse used as substrate.

Supplementary Figure 2 | External morphology of the identified bacterial strains.

Supplementary Figure 3 | Biochemical analysis of the bacterial strains.

Supplementary Figure 4 | CMC hydrolysis of the bacterial strains of CMC-congo red plates.

Supplementary Figure 5 | Phylogenetic tree of the molecularly identified bacterial strains. The phylogenetic tree between 39 taxa was generated using the UPGMA method.

REFERENCES

- Acharya, S., and Chaudhary, A. (2012). Alkaline cellulase produced by a newly isolated thermophilic *Aneurinibacillus thermoaerophilus* WBS2 from hot spring, India. *Afr. J. Microbiol. Res.* 6, 5453–5458. doi: 10.5897/AJMR11.1491
- Agrawal, R., Bhadana, B., Mathur, A. S., Kumar, R., Gupta, R. P., and Satlewal, A. (2018a). Improved enzymatic hydrolysis of pilot scale pretreated rice straw at high total solids loading. *Front. Energy Res.* 6:115. doi: 10.3389/fenrg.2018.00115
- Agrawal, R., Satlewal, A., Mathur, A. S., Gupta, R. P., Raj, T., Kumar, R., et al. (2018b). Kinetic and enzyme recycling studies of immobilized β -glucosidase for lignocellulosic biomass hydrolysis. *Environ. Eng. Manag. J.* 17, 1385–1398. doi: 10.30638/eemj.2018.137
- Agrawal, R., Satlewal, A., Sharma, B., Mathur, A., Gupta, R., Tuli, D., et al. (2017). Induction of cellulases by disaccharides or their derivatives in *Penicillium janthinellum* EMS-UV-8 mutant. *Biofuels* 8, 615–622. doi: 10.1080/17597269.2016.1242692
- Agrawal, R., Semwal, S., Kumar, R., Mathur, A., Gupta, R. P., Tuli, D. K., et al. (2018c). Synergistic enzyme cocktail to enhance hydrolysis of steam exploded wheat straw at pilot scale. *Front. Energy Res.* 6:122. doi: 10.3389/fenrg.2018.00122
- Agrawal, R., Srivastava, A., and Verma, A. K. (2016). Immobilization of β -glucosidase onto silicon oxide nanoparticles and augment of phenolics in sugarcane juice. *J. Food Sci. Technol.* 53, 3002–3012. doi: 10.1007/s13197-016-2269-x
- Al Azkawi, A. S., Sivakumar, N., and Al Bahry, S. (2018). Bioprocessing of cardboard waste for cellulase production. *Biomass Convers. Bior.* 8, 597–606. doi: 10.1007/s13399-018-0309-7
- Ariffin, H., Abdullah, N., Umi Kalsom, M. S., Shirai, Y., and Hassan, M. A. (2006). Production and characterization of cellulase by *Bacillus pumilus* EB3. *Int. J. Eng. Technol.* 3, 47–53.
- Baharuddin, M., Ahmad, A., La Nafie, N., and Zenta, F. (2016). Cellulase enzyme activity of bacillus circulans from larvae *Cossus cossus* in lignocellulosic substrate. *Am. J. Biomed. Life Sci.* 4, 21–25. doi: 10.11648/j.ajbls.20160402.13
- Budihal, S. R., Agsar, D., and Patil, S. R. (2016). Enhanced production and application of acidothermophilic *Streptomyces* cellulase. *Bioresour. Technol.* 200, 706–712. doi: 10.1016/j.biortech.2015.10.098
- Dabhi, B. K., Vyas, R. V., and Shelat, H. N. (2014). Use of banana waste for the production of cellulolytic enzymes under solid substrate fermentation using bacterial consortium. *Int. J. Curr. Microbiol. App. Sci.* 3, 337–346.
- Das, A., Bhattacharya, S., and Murali, L. (2010). Production of cellulase from a thermophilic *Bacillus* sp. isolated from cow dung. *Am. Eurasian J. Agric. Environ. Sci.* 8, 685–691.
- Dipasquale, L., Romano, I., Picariello, G., Calandrelli, V., and Lama, L. (2014). Characterization of a native cellulase activity from an anaerobic thermophilic hydrogen-producing bacterium *Thermosipho* sp. strain 3. *Ann. Microbiol.* 64, 1493–1503. doi: 10.1007/s13213-013-0792-9
- Gupta, A., and Verma, J. P. (2015). Sustainable bio-ethanol production from agro-residues: a review. *Renew. Sust. Energy Rev.* 41, 550–567. doi: 10.1016/j.rser.2014.08.032
- Gupta, P., Samant, K., and Sahu, A. (2012). Isolation of cellulose-degrading bacteria and determination of their cellulolytic potential. *Int. J. Microbiol.* 2012:578925. doi: 10.1155/2012/578925
- Gupta, R., Khasa, Y. P., and Kuhad, R. C. (2011). Evaluation of pretreatment methods in improving the enzymatic saccharification of cellulosic materials. *Carbohydr. Polym.* 84, 1103–1109. doi: 10.1016/j.carbpol.2010.12.074
- Hamilton-Brehm, S. D., Mosher, J. J., Vishnivetskaya, T., Podar, M., Carroll, S., Allman, S., et al. (2010). *Caldicellulosiruptor obsidiansis* sp. nov., an anaerobic, extremely thermophilic, cellulolytic bacterium isolated from Obsidian Pool, Yellowstone National Park. *Appl. Environ. Microbiol.* 76, 1014–1020. doi: 10.1128/AEM.01903-09
- Heck, J. X., Hertz, P. F., and Ayub, M. A. (2002). Cellulase and xylanase productions by isolated Amazon *Bacillus* strains using soybean industrial residue based solid-state cultivation. *Braz. J. Microbiol.* 33, 213–218. doi: 10.1590/S1517-83822002000300005
- Hungate, R. E. (1950). The anaerobic mesophilic cellulolytic bacteria. *Bacteriol. Rev.* 14, 1–49.
- Kato, S., Haruta, S., Cui, Z. J., Ishii, M., and Igarashi, Y. (2005). Stable coexistence of five bacterial strains as a cellulose-degrading community. *Appl. Environ. Microbiol.* 71, 7099–7106. doi: 10.1128/AEM.71.11.7099-7106.2005
- Ko, C. H., Chen, W. L., Tsai, C. H., Jane, W. N., Liu, C. C., and Tu, J. (2007). *Paenibacillus campinasensis* BL11: a wood material-utilizing bacterial strain isolated from black liquor. *Bior. Technol.* 98, 2727–2733. doi: 10.1016/j.biortech.2006.09.034

- Koeck, D. E., Ludwig, W., Wanner, G., Zverlov, V. V., Liebl, W., and Schwarz, W. H. (2015). *Herbinix hemicellulosilytica* gen. nov., sp. nov., a thermophilic cellulose-degrading bacterium isolated from a thermophilic biogas reactor. *Int. J. Syst. Evol. Microbiol.* 65, 2365–2371. doi: 10.1099/ijms.0.000264
- Koeck, D. E., Mechelke, M., Zverlov, V. V., Liebl, W., and Schwarz, W. H. (2016). *Herbivorax saccincola* gen. nov., sp. nov., a cellulolytic, anaerobic, thermophilic bacterium isolated via in sacco enrichments from a lab-scale biogas reactor. *Int. J. Syst. Evol. Microbiol.* 66, 4458–4463. doi: 10.1099/ijsem.0.001374
- Ladeira, S. A., Cruz, E., Delatorre, A. B., Barbosa, J. B., and Leal Martins, M. L. (2015). Cellulase production by thermophilic *Bacillus* sp: SMIA-2 and its detergent compatibility. *Electron J. Biotech.* 18, 110–115. doi: 10.1016/j.ejbt.2014.12.008
- Maki, M., Leung, K. T., and Qin, W. (2009). The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. *Int. J. Biol. Sci.* 5:500. doi: 10.7150/ijbs.5.500
- Miller, G. L., Blum, R., Glennon, W. E., and Burton, A. L. (1960). Measurement of carboxymethylcellulase activity. *Anal. Biochem.* 1, 127–132. doi: 10.1016/0003-2697(60)90004-X
- Mohammad, B. T., Al Daghistani, H. I., Jaouani, A., Abdel-Latif, S., and Kennes, C. (2017). Isolation and characterization of thermophilic bacteria from Jordanian hot springs: *Bacillus licheniformis* and *Thermomonas hydrothermalis* isolates as potential producers of thermostable enzymes. *Int. J. Microbiol.* 2017:6943952. doi: 10.1155/2017/6943952
- Mohanta, Y. K. (2014). Isolation of cellulose-degrading actinomycetes and evaluation of their cellulolytic potential. *J. Biosci. Bioeng.* 2, 1–5. doi: 10.13189/bb.2014.020101
- Nair, A. S., Al-Battashi, H., Al-Akzawi, A., Annamalai, N., Gujarathi, A., Al-Bahry, S., et al. (2018). Waste office paper: a potential feedstock for cellulase production by a novel strain *Bacillus velezensis* ASN1. *Waste Manag.* 79, 491–500. doi: 10.1016/j.wasman.2018.08.014
- Najar, I. N., Sherpa, M. T., Das, S., Verma, K., Dubey, V. K., and Thakur, N. (2018). *Geobacillus yumthangensis* sp. nov., a thermophilic bacterium isolated from a north-east Indian hot spring. *Int. J. Syst. Evol. Microbiol.* 68, 3430–3434. doi: 10.1099/ijsem.0.003002
- Park, J. I., Steen, E. J., Burd, H., Evans, S. S., Redding-Johnson, A. M., and Batth, T. (2012). A thermophilic ionic liquid-tolerant cellulase cocktail for the production of cellulosic biofuels. *PLoS ONE* 7:e37010. doi: 10.1371/journal.pone.0037010
- Rastogi, G., Muppidi, G. L., Gurram, R. N., Adhikari, A., Bischoff, K. M., Hughes, S. R., et al. (2009). Isolation and characterization of cellulose-degrading bacteria from the deep subsurface of the Homestake gold mine, Lead, South Dakota, USA. *J. Ind. Microbiol. Biotechnol.* 36, 585–598. doi: 10.1007/s10295-009-0528-9
- Rawway, M., Ali, S. G., and Badawy, A. S. (2018). Isolation and identification of cellulose degrading bacteria from different sources at Assiut governorate (Upper Egypt). *J. Ecol. Heal. Environ. Int. J.* 6, 15–24. doi: 10.18576/jeh/060103
- Robert, C., and Bernalier-Donadille, A. (2003). The cellulolytic microflora of the human colon: evidence of microcrystalline cellulose-degrading bacteria in methane-excreting subjects. *FEMS Microbiol. Ecol.* 46, 81–89. doi: 10.1016/S0168-6496(03)00207-1
- Russell, D. W., and Sambrook, J. (2001). *Molecular Cloning: A Laboratory Manual*. New York, NY: Cold Spring Harbor Laboratory.
- Satlewal, A., Agrawal, R., Bhagia, S., Sangoro, J., and Ragauskas, A. J. (2018a). Natural deep eutectic solvents for lignocellulosic biomass pretreatment: recent developments, challenges and novel opportunities. *Biotechnol. Adv.* 36, 2032–2050. doi: 10.1016/j.biotechadv.2018.08.009
- Satlewal, A., Agrawal, R., Das, P., Bhagia, S., Pu, Y., Puri, S. K., et al. (2018b). Assessing the facile pretreatments of bagasse for efficient enzymatic conversion and their impacts on structural and chemical properties. *ACS Sustain. Chem. Eng.* 7, 1095–1104. doi: 10.1021/acssuschemeng.8b04773
- Shareef, I., Satheesh, M., and Christopher, S. X. (2015). Isolation and identification of cellulose degrading microbes. *Int. J. Innov. Res. Sci. Eng.* 8, 6788–6793. doi: 10.15680/IJIRSET.2015.0408012
- Sharma, B., Agrawal, R., Singhania, R. R., Satlewal, A., Mathur, A., Tuli, D., et al. (2015). Untreated wheat straw: potential source for diverse cellulolytic enzyme secretion by *Penicillium janthinellum* EMS-UV-8 mutant. *Bioresour. Technol.* 196, 518–524. doi: 10.1016/j.biortech.2015.08.012
- Sivakumar, N., Al Zadjali, A., Al Bahry, S., Elshafie, A., and Eltayeb, E. A. (2016). Isolation and characterization of cellulolytic *Bacillus licheniformis* from compost. *Afr. J. Biotechnol.* 15, 2434–2446. doi: 10.5897/AJB2016.15641
- Srivastava, N., Srivastava, M., Mishra, P. K., Gupta, V. K., Molina, G., Rodriguez-Couto, S., et al. (2018). Applications of fungal cellulases in biofuel production: advances and limitations. *Renew. Sust. Energ. Rev.* 82, 2379–2386. doi: 10.1016/j.rser.2017.08.074
- Strahsburger, E., de Lacey, A. M. L., Marotti, I., DiGioia, D., Biavati, B., and Dinelli, G. (2017). *In vivo* assay to identify bacteria with β -glucosidase activity. *Electron J. Biotechnol.* 30, 83–87. doi: 10.1016/j.ejbt.2017.08.010
- Tabassum, F., Irfan, M., Shakir, H. A., and Qazi, J. I. (2018). RSM based optimization of nutritional conditions for cellulase mediated Saccharification by *Bacillus cereus*. *J. Biol. Eng.* 12:7. doi: 10.1186/s13036-018-0097-4
- Verma, J. P., Jaiswal, D. K., Krishna, R., Prakash, S., Yadav, J., and Singh, V. (2018). Characterization and screening of thermophilic *Bacillus* strains for developing plant growth promoting consortium from hot spring of Leh and Ladakh region of India. *Front. Microbiol.* 9:1293. doi: 10.3389/fmicb.2018.01293
- Yadav, A. N., Verma, P., Kumar, M., Pal, K. K., Dey, R., Gupta, A., et al. (2015). Diversity and phylogenetic profiling of niche-specific *Bacilli* from extreme environments of India. *Ann. Microbiol.* 65, 611–629. doi: 10.1007/s13213-014-0897-9
- Zhang, Y. H. P., Hong, J., and Ye, X. (eds.). (2009). “Cellulase assays,” in *Biofuels* (Totowa, NJ: Humana Press), 211–213.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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