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A Report on Metal Enhanced, Solvent Tolerant Endoglucanase from Strict Thermophilic *Bacillus* sp. PW1 and *Bacillus* sp. PW2 of North West Himalayas

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ABSTRACT: The cellulolytic activity of two thermophilic bacterial strains *Bacillus* sp. PW1 and *Bacillus* sp. PW2 isolated from Tattapani hot spring of North Western Himalayas were characterized. Both the strains exhibited extracellular CMCase activity, indicating the endoglucanase potential of the cellulases. There was no detectable cellulase activity of both the strains below 40°C, indicating thermophilic nature of the cellulase enzymes. Optimum temperature and pH for cellulase activity of both PW1 and PW2 was 80°C and 8, respectively. Various metal ions enhanced the cellulase activity of the bacterial strains, with Ca^{2+}, and Hg^{2+} showing the maximum effect. Interestingly, the cellulase of the two bacterial strains was active in the presence of solvents (0.5 % v/v) like Ethanol, n-butanol and cyclohexane, and the denaturant, SDS (0.5% w/v). Glucose and Galactose were best carbon sources, while beef extract and tryptone the best nitrogen sources for the maximum production of cellulase by the two bacterial strains. Zymogram analysis indicated the presence of two proteins bands in the crude cellulase of *Bacillus* sp. PW1.

Keywords: Endoglucanase, Thermophilic, metal ions, organic solvent tolerance, Tattapani, hot spring, Himachal Pradesh, India.

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INTRODUCTION

Cellulases encompass a group of enzymes that hydrolyze β -1,4 linkages in cellulose chains to release monomeric chains of glucose. In nature, complete cellulose hydrolysis is mediated by a combination of three types of cellulases: endoglucanases (EC 3.2.1.4), exoglucanases, including cellobiohydrolases (CBHs) (EC 3.2.1.91), and β-glucosidases (BG) (EC 3.2.1.21). They are primarily produced by fungi, and few members of bacteria, protozoans, plants, and animals (Kuhad, *et al.*, 2011). The catalytic modules of cellulases are classified into numerous families based on their amino acid sequences and crystal structures (Henrissat, *et al.*, 1991). Most cellulases harbor a non-catalytic carbohydrate-binding module (CBM) and/or other functionally known or unknown modules, which may be located at the N- or C-terminus of a catalytic module. Endoglucanases, or CMCase, randomly cut β -1, 4-bonds of cellulose chains, generating new ends. Different endoglucanases are produced by archaea, bacteria, fungi, plants, and animals with different catalytic modules belonging to families 5–9, 12, 44, 45, 48, 51, and 74 (Cohen, *et al.*, 2005; Wilson, *et al.*, 2008; Mejia, *et al.*, 2008; Parsiegla, *et al.*, 2008; Yoon, *et al.*, 2008; Zverlov, *et al.*, 2005).

To hydrolyze and metabolize insoluble cellulose, the microorganisms secrete cellulases that are either free or cell-surface-bound in the form of cellulosomes (Kuhad, *et al.*, 2011). Cellulases are used for a large variety of industrial purposes, including textile, pulp and paper, food, and pharma industries, as well as additives in detergents and animal feeds (Bayer *et al.*, 2007; Himmel, *et al.*, 1999; Zaldivar, *et al.*, 2001). The growing market for industrial enzymes demands for catalytically efficient and thermostable enzymes in most applications (Bruins, *et al.*, 2001; Elleuche, *et al.*, 2015). Thermozymes, enzymes produced by extremophiles called thermophiles possess the unique property of thermo stability and efficient catalysis under conditions of high temperature (Bruins, *et al.*, 2001; Elleuche, *et al.*, 2015). Moreover, thermozymes exhibit catalytic potential in organic media, which is ideal for several industrial applications (Elleuche, *et al.*, 2015). Thus, cellulases from thermophiles are being explored as an efficient source for industrial applications (Thankappan, *et al.*, 2018). The Himalayan range possesses an array of thermal hot springs, some of which have been explored for thermophilic microbes and their hydrolytic enzymes (Sahay *et al.*, 2017; Sharma, *et al.*, 2015; Thankappan, *et al.*, 2018).

Previously, we reported the isolation and characterization of cellulase from thermophiles of the hot spring located in Tattapani, Himachal Pradesh, India (Sharma, *et al.,* 2015). The extracellular thermophilic cellulase produced by *Geobacillus* sp. was found to be thermostable, and tolerant to several organic solvents (Sharma, *et al.,* 2015). In addition, two celluloytic thermophiles namely *Bacillus*sp. PW1 (Genbank Acc no. KU711837) and *Bacillus* sp. PW2 (Genbank Acc no. KU711838) were found to possess dual function endoglucanase gene of M42 aminopeptidase/ endoglucanase family (Sharma, *et al.,* 2019). In this study, we report the biochemical characterization of the endoglucanase activity of *Bacillus* sp. PW1 and *Bacillus* sp. PW2 towards biotechnological applications.

MATERIAL AND METHODS

A. Strains used in study

Two cellulolytic thermophilic bacterial strains namely *Bacillus* sp. PW1 (Acc no. KU711837) and *Bacillus* sp. PW2 (Genbank Acc no. KU711838) isolated from water samples of hot spring located in Tattapani, district Mandi, Himachal Pradesh, India were used in this study. The two bacterial strains were cultured in nutrient broth (NB; Himedia Labs, India) at 60ºC.

B. Cellulase Assay

Bacillus sp. PW1 and *Bacillus* sp. PW2 were cultured in nutrient broth at 60°C for 48 hours (A_{600} ~ 2.0). The cell free supernatants were harvested and used as the source of cellulase. Cellulase activity was measured by the DNS method (Miller, *et al.,* 1959), through the determination of the amount of reducing sugars liberated from carboxy methylcellulose (CMC) at 540 nm. 20µg of total protein as crude cell free enzyme was added to 0.2 ml of 1% CMC in 0.1 M sodium phosphate buffer, pH 7. The reaction mixture was incubated at 60°C for 30 min and the reaction was stopped by the addition of 2.0 ml DNS reagent. Enzyme activity was calculated as the amount of enzyme liberating 1µg of glucose. Enzyme blank (EB) and substrate blank (SB) were included in the assays, with only added enzyme source or substrate, respectively.

C. Effect of physical and chemical parameters on extracellular cellulase activity of PW1 and PW2 thermophilic bacterial isolates

Determination of optimal pH and temperature for cellulase enzyme activity. Optimum temperature for cellulase activity was determined by incubating the reaction mixture at different temperatures ranging from 30 to 90°C. Effect of pH on the cellulase activity was studied by adjusting the pH of reaction buffer using citrate – phosphate buffer (pH 5 and 6), phosphate buffer (pH 7) and Tris- HCl buffer (pH 8, 9 and 10).

Effect of carbon and nitrogen sources on the cellulase activity of thermophilic bacterial isolates. *Bacillus* sp.PW1 and *Bacillus* sp. PW2 were cultured in M9 medium supplemented with 1% of respective carbon source (glucose, starch, sucrose, fructose, trehalose, glycerol, lactose, raffinose, galactose and sorbitol) or 0.25% of respective nitrogen source (yeast extract, peptone, NH_4Cl, tryptone, beef extract, casein acid hydrolysate, and urea) at 60°C for 48 h (A_{600} ~ 1.0). Cellulase activity in each sample was determined as described above.

Effect of chaotropic agents, metal ions and solvents on cellulase activity of thermophilic bacterial isolates. The enzymatic assays were carried out as described above, except that different concentrations (1, 5 and 10 mM) of metal ions such as nickel ($NiSO_4.6H_2O$), zinc ($ZnSO_4.H_2O$), cobalt ($Co(NO_3)_2.6H_2O$), mercury ($HgCl_2$), manganese ($MnSO_4.H_2O$), ferrous ($FeSO_4.7H_2O$), calcium ($CaCl_2$), magnesium ($MgCl_2$) and copper ($CuSO_4$) were included in the reaction. Similarly, the effect of solvents (ethanol, phenol, n- butanol, cyclohexane, hydrogen peroxide, pyridine and toluene), and detergents (sodium dodecyl sulphate (SDS), triton X-100) was tested in the cellulase assay.

D. Activity staining of Cellulase

Ammonium sulphate (60-90% saturation) was added to the cell free supernatant of *Bacillus* sp. PW1 and precipitate was collected by centrifugation. The precipitate was resuspended in 0.01 M sodium phosphate buffer (pH 7) and subjected to native and SDS-PAGE. For activity staining, the protein samples (20 µg total protein) were mixed with the sample buffer without any reducing/ denaturing agent and resolved on 12% separating native gel containing 0.1% (w/v) CMC. After electrophoresis, gel was soaked in 0.1 M sodium phosphate buffer, pH 7.0 followed by staining in 0.2% Congo red for 1 h and destained with 2 M NaCl until the clear red bands were observed. SDS-PAGE was performed according to Laemmli, *et al.,* 1970 using 12% separating gel. Subsequent to electrophoresis, the gel was stained with 0.15% Coomassie Brilliant Blue (CBB) R-250 followed by destaining.

RESULTS

In our previous study, two thermophilic bacteria *Bacillus* sp.PW1 and *Bacillus* sp. PW2 were found to possess profound extracellular cellulase activity (Sharma, *et al.,* 2019). The biochemical characteristics of the cellulase from the two bacterial strains were studied and are elaborated below.

A. Optimization of physical parameters (pH and temperature) for cellulase activity

The cellulase activity of *Bacillus* sp. PW1 and *Bacillus* sp. PW2 was analyzed at varying pH and temperature of the enzymatic reaction. Optimum pH and temperature for the bacterial isolates *Bacillus* sp. PW1 and *Bacillus* sp. PW2 was found to be pH 8 and 80°C, respectively (Fig. 1). PW1 showed 1295 and 1359.3 U $mg^{-1}min^{-1}$ cellulase activity at pH 8 and 80°C, respectively.

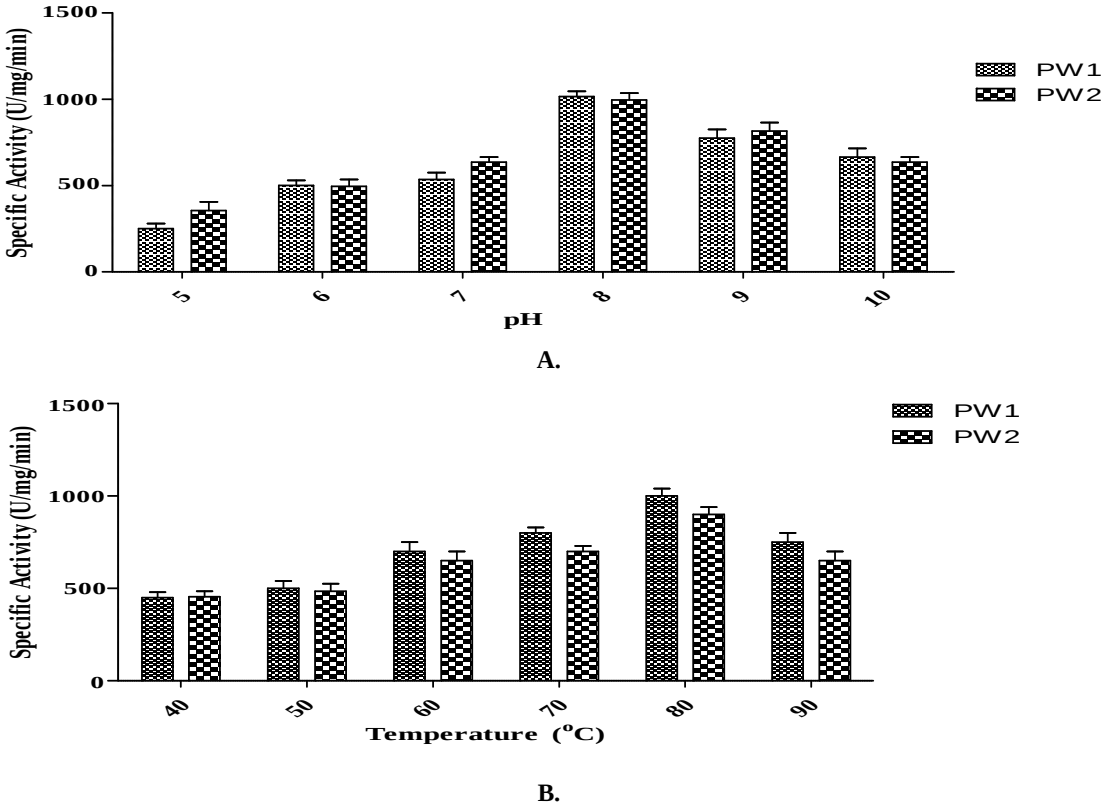


Fig. 1. Effect of temperature and pH on cellulase activity of *Bacillus* sp. PW1 and *Bacillus* sp. PW2. Cellulase assays were carried by incubating the reaction mixtures at varying pH and temperatures. Cell free spent medium was used as a source of cellulase and specific cellulase activity (U $mg^{-1}min^{-1}$) of PW1 and PW2 was plotted against pH (A) and temperature (B) of the enzymatic reaction. Data of three independent experiments was plotted with standard deviation.

PW2 showed 1168, and 1216.3 U $mg^{-1}min^{-1}$ of cellulose activity at pH 8 and 80°C, respectively (Fig. 1 A &B). Studies on the temperature regime of the PW1 and PW2 cellulase activity showed that there was no significant activity below 40°C, indicating thermophilic nature of the enzyme in both cases. Consistent with this observation, cellulose activity of both PW1 and PW2 increased with increasing temperatures from 40-70°C and reached maximum at 80°C (Fig. 1B). About 65-75% activity was retained up to 90°C for both PW1 and PW2, further validating the thermostable nature of the cellulase activity.

B. Effect of carbon and nitrogen sources on the cellulase activity of thermophilic bacterial isolates

To study the best carbon and nitrogen sources in the growth media for maximum production of cellulase, PW1 and PW2 were grown in media with different carbon/nitrogen sources. Among different carbon sources tested, galactose was found to be the best carbon source for production of cellulase by both PW1 and PW2 followed by glucose (Fig. 2A). Cellulase activity of PW1 and PW2 decreased by 5-25% when the bacteria were cultured in the presence of all the other carbon sources tested, when compared to minimal salt medium supplemented with glucose.

Beef extract and tryptone were found to be the best nitrogen source for production of cellulase by PW1 and PW2, respectively (Fig. 2B). Cellulase activity of PW1 and PW2 was decreased by 5-20 % in presence of other nitrogen sources when compared to minimal salt medium supplemented with ammonium chloride.

C. Effect of solvents on cellulase activity of thermophilic bacterial isolates

To study the biotechnological application of PW1 and PW2 cellulase in industrial processes involving organic solvents and oxidants, the enzymatic assays were performed in the presence of common industrial solvents, including phenol, pyridine, ethanol, cyclohexane, H_2O_2, toluene and n-butanol. More than 50 % cellulase activity of both PW1 and PW2 was retained in the presence of all solvents (except H_2O_2 for PW1) at 0.5% (Fig. 3). Interestingly, cellulase activity was enhanced by 1-5% in the presence of ethanol and n-butanol for PW1 and 0.5% ethanol and 0.5% cyclohexane in case of PW2 (Fig. 3). On the other hand, cellulase activity decreased (10-50 %) in the presence of 0.5% of phenol, pyridine, cyclohexane and toluene for PW1 and 0.5% of phenol, pyridine and toluene for PW2 (Fig. 3). Moreover, cellulase activity of both PW1 and PW2 decreased significantly (70-80%) in the presence of H_2O_2.

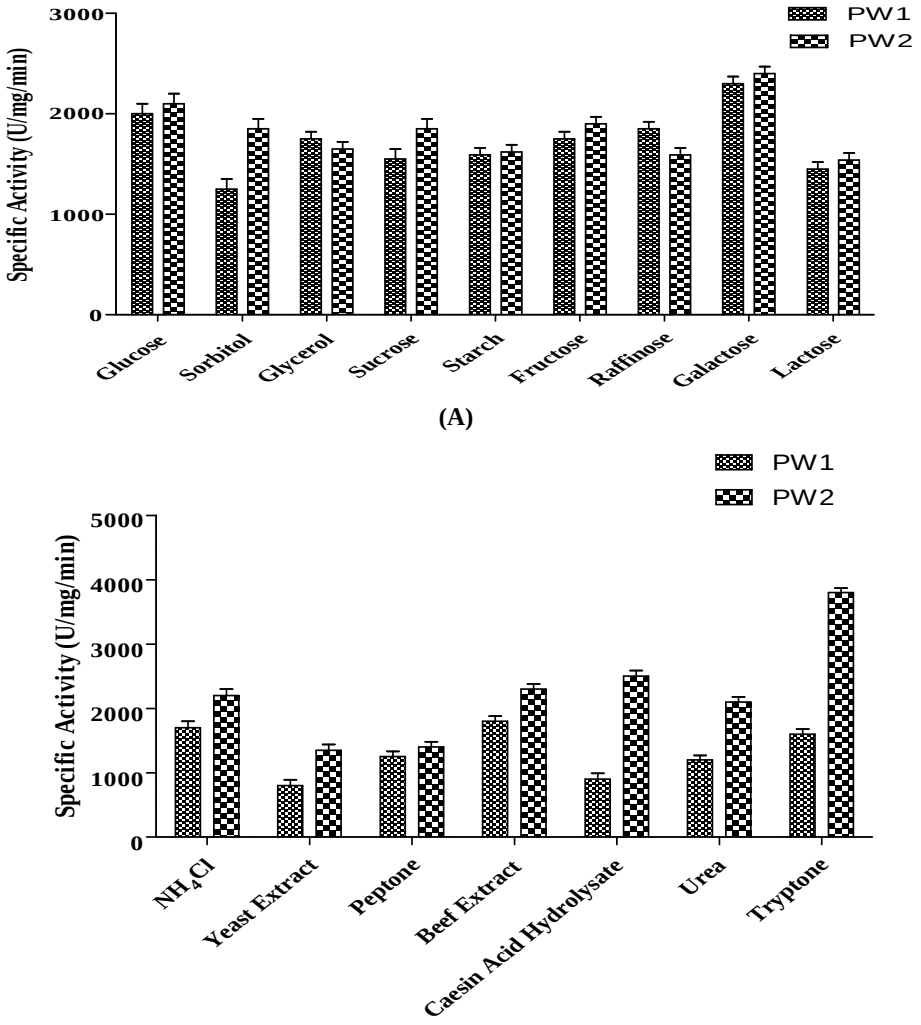


Fig. 2. Effect of carbon and nitrogen sources on cellulase activity of *Bacillus* sp. PW1 and *Bacillus* sp. PW2. The two bacterial strains were cultured in minimal media containing the indicated carbon/ nitrogen source and cell free spent medium was used as a source of cellulase. The specific activity (U $mg^{-1}min^{-1}$) of PW1 and PW2 was plotted against indicated carbon sources (A) and nitrogen sources (B) supplemented in the growth medium. Data of three independent experiments was plotted with standard deviation.

D. Effect of metal ions on cellulase activity of thermophilic bacterial isolates

To identify enhancers of cellulase activity of PW1 and PW2, enzyme assay was carried out by incubating reaction mixture with salts of different metal ions (Ni^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}) and relative cellulase activity was determined. Significantly, all the metal ions enhanced the cellulase activity of both *Bacillus* sp. PW1 and *Bacillus* sp. PW2 in a dose dependent manner upto the concentrations tested (1–10 mM) (Fig. 4). Hg^{2+} and Ca^{2+} were the best enhancers of cellulase activity for *Bacillus* sp. PW1 and *Bacillus* sp. PW2, respectively (Fig. 4).

E. Effect of detergents on cellulase activity of thermophilic bacterial isolates

The potential of cellulase of to resist denaturants action was studied by performing enzyme assays in the presence of SDS and Triton X-100. Cellulase activity of *Bacillus* sp. PW1 was enhanced by 1.8 and 1.6 fold in the presence of 0.1% and 0.5% SDS, respectively (Fig. 5A). Cellulase activity of *Bacillus* sp. PW2 was enhanced by 1.5 and 1.4 fold in the presence of 0.1% and 0.5% SDS, respectively. However, decrease in cellulase activity was observed in the presence of 1% SDS for both PW1 and PW2 (Fig. 5A).

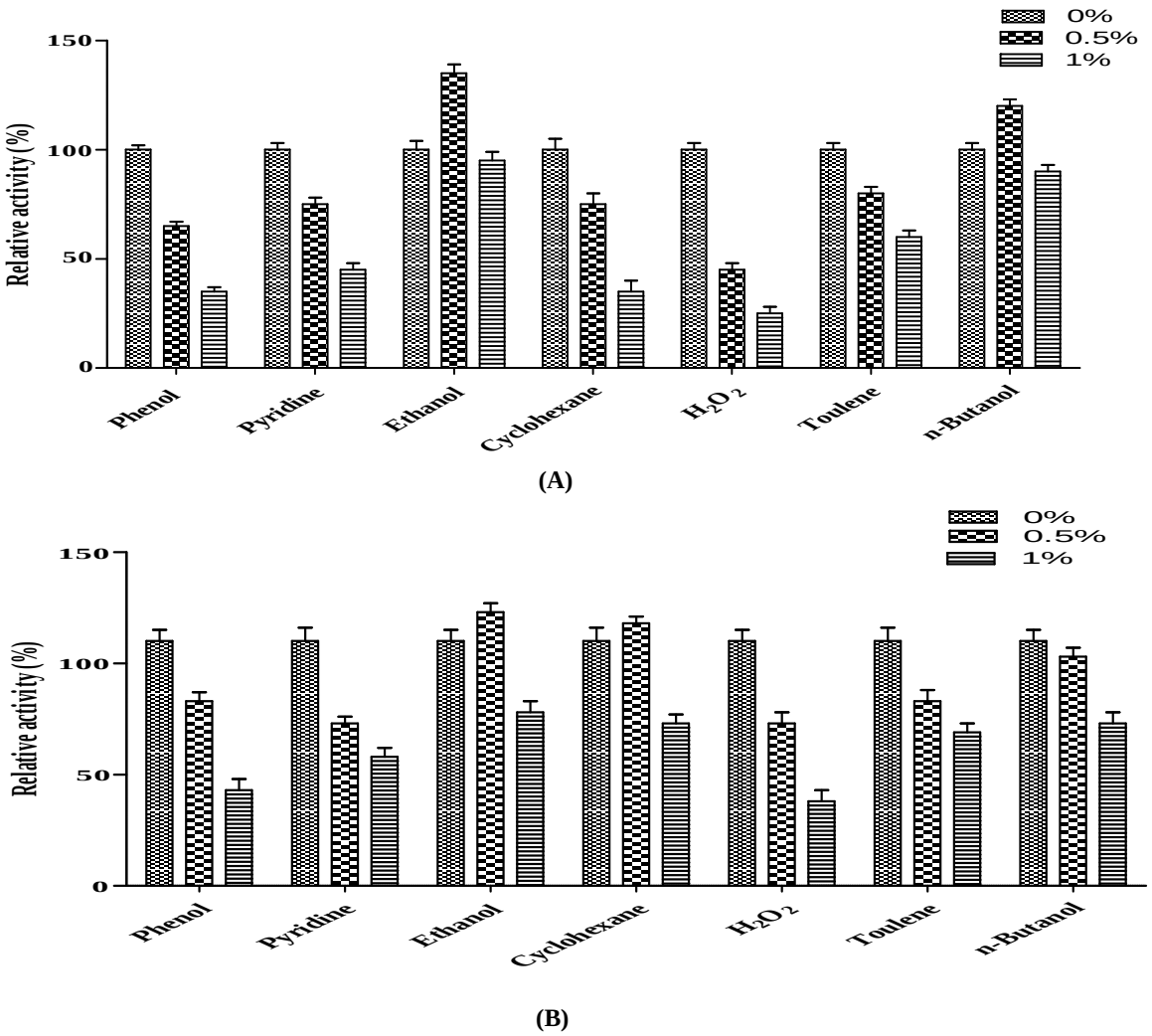


Fig. 3. Effect of organic solvents and oxidant on the cellulase activity of *Bacillus* sp. PW1 and *Bacillus* sp. PW2. Cellulase assays were carried out in the absence and presence of different solvents (0.5 and 1%). Reaction without solvents was considered as 100 % cellulase activity. The relative cellulase activity of *Bacillus* sp. PW1 (A), and *Bacillus* sp. PW2 (B) was plotted against the indicated concentration of solvents and oxidizing agent H_2O_2. Data of three independent experiments was plotted with standard deviation**.**

Cellulase activity of *Bacillus* sp. PW1 and *Bacillus* sp. PW2 decreased by 35-95% in the presence of 0.1–1 % Triton X-100 (Fig. 5B). These results indicate that cellulase activity of both PW1 and PW2 is resistant to SDS (upto 0.5 %) but not Triton X-100.

F. Activity staining of Cellulase Enzyme of Bacillus sp. PW1

Since *Bacillus* sp. PW1 showed higher cellulase activity than *Bacillus* sp. PW2, *Bacillus* sp. PW1 cellulase was selected for activity staining. Cell free supernatant (200 ml) was subjected to ammonium sulphate precipitation at 0-30%, 30-60% and 60-90% concentration. A precipitate was obtained with 60-90% ammonium sulphate fractionation. Therefore the 60-90% precipitate (20µg of protein) was subjected to denaturing and native PAGE analysis. Upon incubation of the native PAGE gel in CMC followed by staining, two prominent bands were detected in 60-90% fraction, indicating them to possess cellulase activity *in situ* (Fig. 6A). SDS-PAGE analysis of the same fraction revealed the presence of multiple bands in the 60-90% fraction corresponding to size of ~ 30 kDa to ~ 100 kDa (Fig. 6B). Further studies are required to decipher the size and composition of the protein/s with cellulase activity.

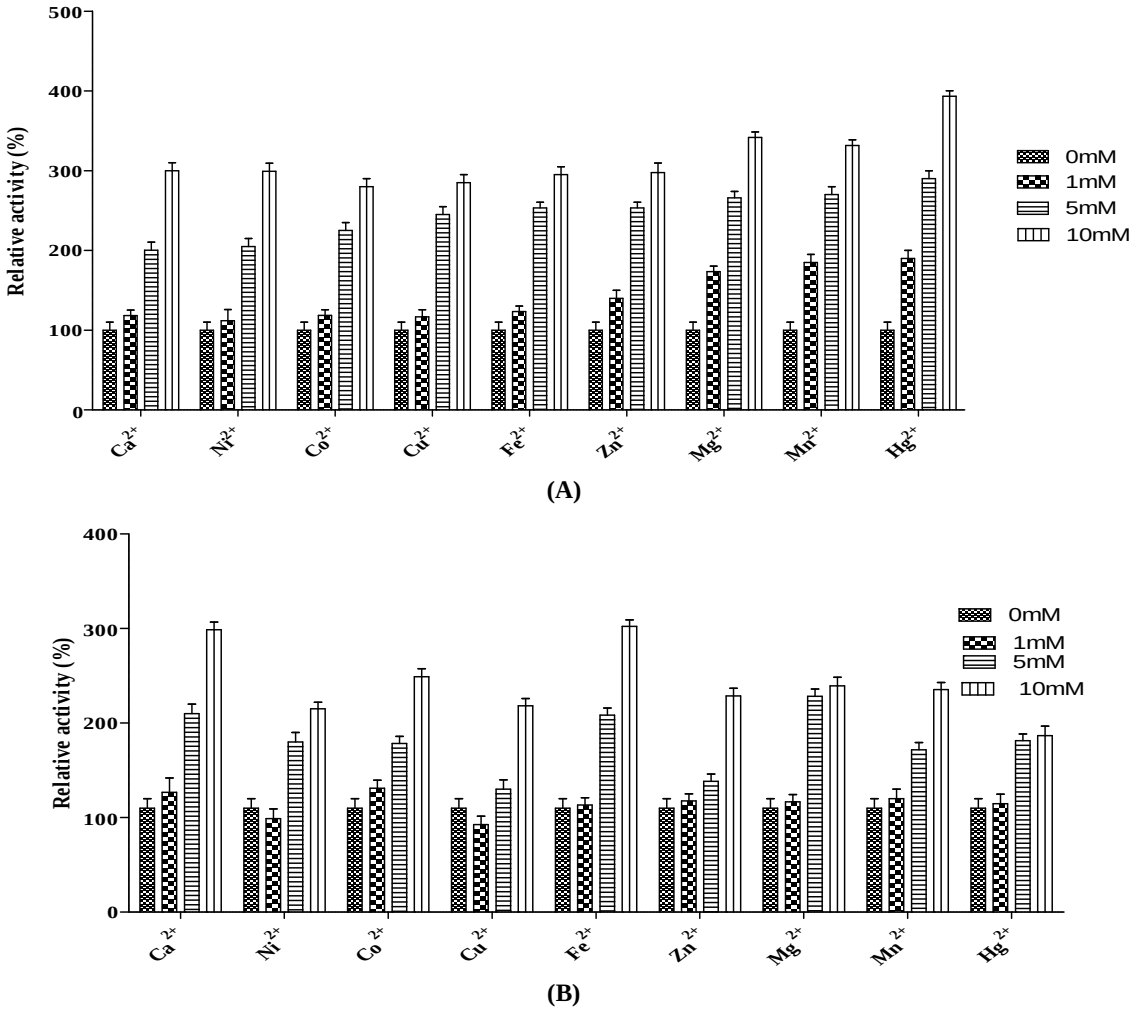
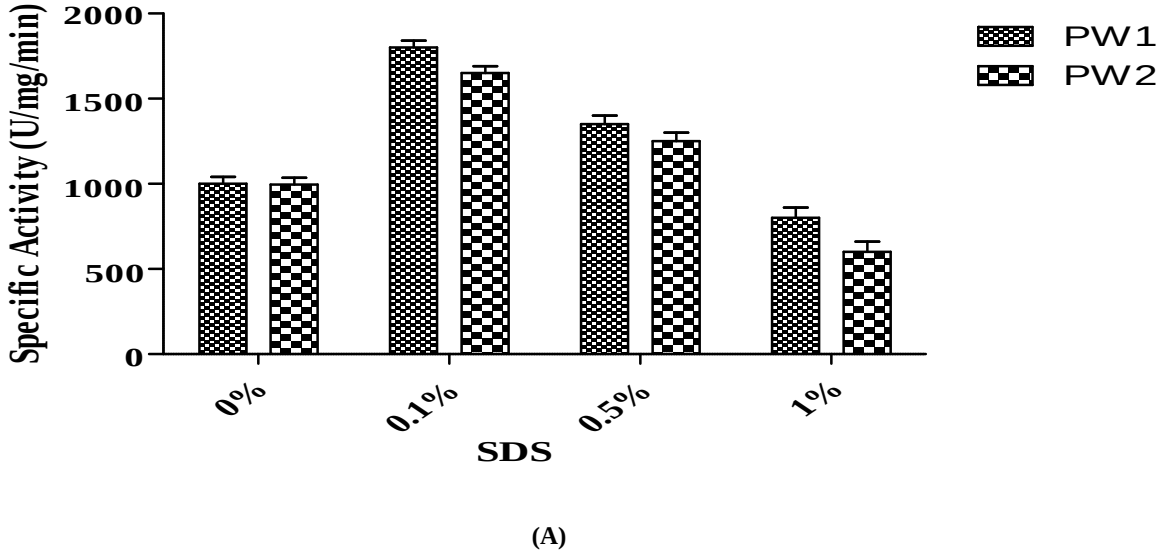
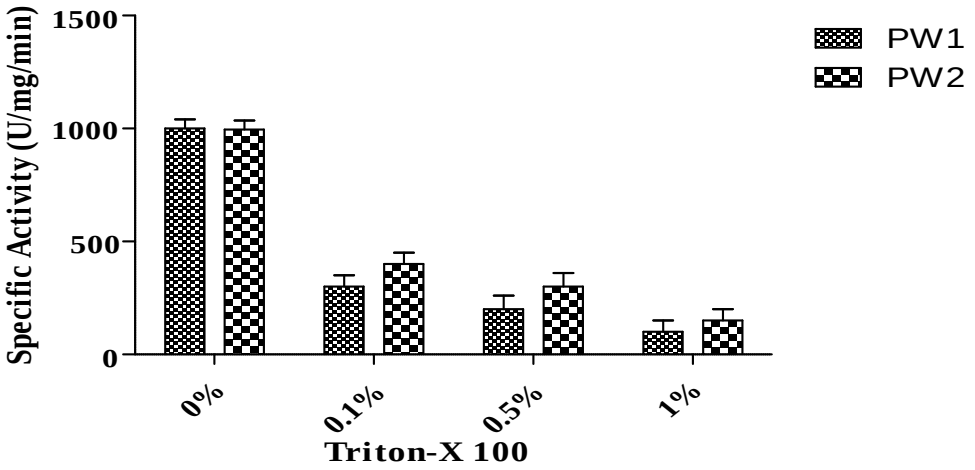


Fig. 4. Effect of metal ions on the cellulase activity of *Bacillus* sp. PW1 and *Bacillus* sp. PW2. Cellulase assays were carried by supplementing reaction mixture with salts of metal ions (Ca^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+} and Hg^{2+}) at 1, 5, and 10 mM. The relative cellulase activity of *Bacillus* sp. PW1 (A), and PW2 (B) with respect to activity without any added metal ions was plotted against the various concentrations of metal ions as indicated. Data of three independent experiments was plotted with standard deviation.





(B)

Fig. 5. Effect of detergents on the cellulase activity of *Bacillus* sp. PW1 and *Bacillus* sp. PW2. Cellulase assays were carried out by supplementing reaction mixture with the following detergents: SDS (panel A) and Triton X-100 (panel B). The specific cellulase activity of *Bacillus* sp. PW1and *Bacillus* sp. PW2 isolates was plotted against the concentrations (% w/v for SDS and % v/v for Triton X-100) of detergents as indicated. Data of three independent experiments was plotted with standard deviation.

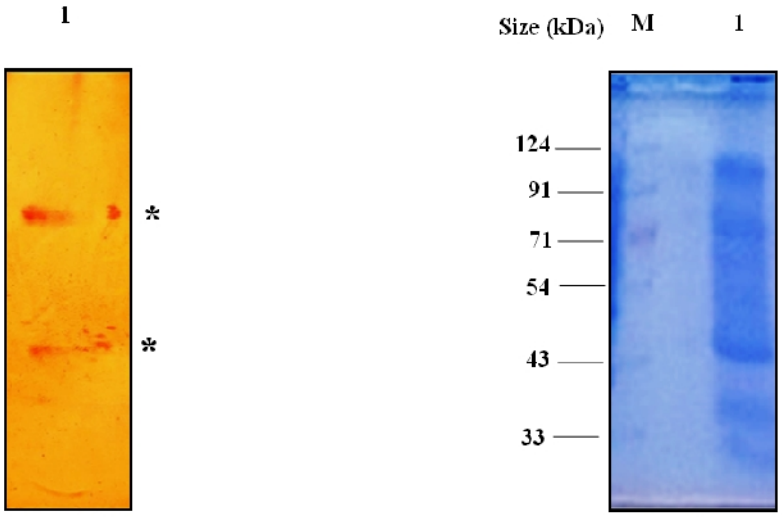


Fig. 6. Zymogram analysis of extracellular fraction of *Bacillus* sp. PW1 for cellulase activity. A. Activity staining of 60-90% ammonium sulphate fraction of *Bacillus sp*. PW1 crude cellulase. Red colored bands corresponding to active cellulase are indicated by asterisk**.** B. SDS-PAGE analysis of 60-90% ammonium sulphate fraction of *Bacillus sp*. PW1 crude cellulose visualized by Coomassie (CBBR-250) dye staining. The size of marker bands is indicated in kDa.

DISCUSSION

Thermophilic microbes offer a unique source of enzymes called thermozymes, which are beneficial for several biotechnological applications. In the present study, two thermophiles *Bacillus* sp. PW1 and *Bacillus* sp. PW2 isolated from Tattapani hot spring Himachal Pradesh were studied for extracellular cellulase production. The extracellular cellulase from *Bacillus* sp.PW1 and *Bacillus* sp. PW2 were able to liberate glucose from CMC, indicating the endoglucanase potential of the enzyme produced. The bacterial isolates showed extracellular cellulase activity in a pH range of 6-8 and temperature 60°C-80°C, but maximum cellulase activity was obtained at pH 8 and 80°C. Thermophilic cellulases have been reported from different locations worldwide (Ibrahim, *et al.*, 2007; Liang, *et al.*, 2014; Sahay, *et al.*, 2017; Sharma, *et al.*, 2015; Thankappan, *et al.*, 2018).

However, there are few studies on the metal dependence and solvent tolerance of thermophilic cellulases in the literature (Sharma, *et al.,* 2015; Thankappan, *et al.,* 2018). Tolerance to organic solvents and heavy metals provides beneficial properties to industrial enzymes owing to their potential applications in bioremediation (Garg, *et al.,* 2015; Ramya, *et al.,* 2018). In the present study, most metal ions enhanced the endoglucanase activity of *Bacillus* sp. PW1 and *Bacillus* sp. PW2, with Hg^{2+} and Ca^{2+} being the best activators. Moreover, the endoglucanse activity of both strains showed tolerance to solvents like ethanol, n-butanol and cyclohexane, and moderate levels of detergents like SDS. In another study, the cellulase activity of *Bacillus licheniformis* KBFB3 was reported to be enhanced in the presence of Ca^{2+}, whereas moderately inhibited by Cu^{2+}, Zn^{2+}, urea, SDS and H_2O_2 (Thankappan, *et al.,* 2018). An organic solvent-tolerant cellulose of 47 kDa was reported from *Bacillus amyloliquefaciens* AK9, Pakistan (Irfan, *et al.,* 2017). Although two cellulase active bands were detected in zymogram analysis of crude enzyme of *Bacillus* sp. PW1, the exact size of the cellulase needs to be determined.

CONCLUSION

In our study, we have explored two thermophiles namely *Bacillus* sp. PW1 and *Bacillus* sp. PW2 of Tattapani hot spring (Himachal Pradesh, India) for their cellulolytic potential. Extracellular Cellulase activity of the two bacterial strains was analyzed at various pH and temperature. Maximum activity was observed at pH 8 and 80ºC, indicating thermoalkalophilic nature of the cellulase activity of *Bacillus* sp. PW1 and *Bacillus* sp. PW2. Production of cellulase by both the strains was best in media containing Glucose and Galactose as carbon sources, and beef Extract, and tryptone as nitrogen sources. Various metal ions enhanced the cellulase activity of both the bacterial strains, with Hg^{2+} and Ca^{2+} showing the best activation of activity. The cellulase activity of both strains showed tolerance to a wide range of solvents and detergents like SDS, thereby revealing their ample potential for industrial applications. Thus, the thermozymes of *Bacillus* sp. PW1 and *Bacillus* sp. PW2 characterized in the present study have great application in industries requiring thermostable, solvent tolerant cellulase activities.

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

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