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Research Article

Characterization of extracellular thermophillic cellulase from thermophilic Geobacillus sp. isolated from Tattapani Hot spring of Himachal Pradesh, India.

Parul Sharma, Sonika Gupta,

Anuradha Sourirajan and Kamal Dev*

Faculty of Applied Sciencess & Biotechnology,
Shoolini University, Solan, Himachal Pradesh, India, Postal Code: 173212
*Corresponding Author: Email: kdb_nih@yahoo.com, kamaldev@shooliniuniversity.com

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ABSTRACT:

Geobacillus kaustrophilus PW11, Geobacillus toebii PW12, Geobacillus thermoleovorans PW13 and Geobacillus toebii PS4 were isolated form Tattapani hotspring of Himachal Pradesh, India and characterized for extracellular cellulase activity. All the four Geobacillus spp. exhibited thermophilic cellulase activity, which was predominantly extracellular. The activity was optimum at 80- 90°C and pH 6 - 8.0. Interestingly, no cellulase activity was observed at temperature less than 40°C, indicating its thermophilic nature. The cellulase enzyme produced by PW11, PW12, PW13 and PS4 was thermostable and exhibited activity even at 100°C. Among the metal ions tested, Mn2+, Co2+ and Fe2+ significantly enhanced the cellulase activity of PW11, PW12 and PW13 by 2-5 folds. Hg2+ (1mM) strongly inhibited enzyme activity of PW11, PW13 and PS4, enzyme activity of PW12 was slightly enhanced in the presence of Hg2+ (1mM) and Cd2+ (5Mm). The cellulase activity of all isolates was increased by 6 – 39 % in the presence of 0.1 % SDS and Triton X100. Enzyme activity of PW11 was increased by 1.7 folds in the presence of 5 mM EDTA and PW12, PW13 and PS4 showed increase in cellulase activity by 1.4, 1.6 and 1.3 folds in the presence of 1 mM EDTA respectively. The thermophilic cellulase activities of PW11, PW12, PW13 and PS4 are highly advantageous for downstream industrial applications.

Key words: Thermophilic, Cellulase, Extracellular, Geobacillus, Tattapani, Hotspring

INTRODUCTION

Thermophiles are the group of extremophiles that requires high temperature for growth and survive. The hydrolytic enzymes (lipases, cellulases, proteases, chitinases, xylanases, pullulanases) produced by them are known as thermozymes. Enzymes from thermophilic microorganisms are active and stable at high elevated temperatures as well as resistant to chemical denaturation.

Cellulose is the most abundant organic compound on earth and has extensively used as a substrate for the production of single cell proteins, biofuels, and various other chemicals through microbial enzymatic degradation. The conversion of cellulosic biomass to fermentable sugars requires, different types of cellulose namely, β-1,4 endoglucanase (EC 3.4.1.4), β-1,4 exoglucanase (EC 3.2.1.91) and β-1,4 glucosidase (EC 3.2.1.21)) [1]. Various bacteria, fungi and yeast synthesize these enzymes, but the most extensively studied cellulases are those produced by efficient

lignocellulose degrading fungi, particularly *Trichoderma* and *Aspergillus* [2-3]. In nature, fungi tend to produce more cellulases than bacteria, however, cellulases produced by bacteria are better catalyst as they encounter less feedback inhibition. The bacterial cellulases are thermostable and also active at an alkaline pH in comparison to fungal cellulases [4]. The optimal activity of thermophilic cellulolytic *Bacillus* strains from Bakreshwar hot spring, India was observed at 60°C [5]. Endocellulase, with the ability to hydrolyze microcrystalline cellulose, was isolated from the extremely thermophilic bacterium *Anaerocellum thermophilum* and maximal activity was observed at pH 5.0-6.0 and 85-950C. Thermostable cellulases from archaea (*Pyrococcus horikoshi*) [6] and bacteria (*Geobacillus pallidus*) has been reported [7]. The thermophilic cellulases of *Clostridium thermocellum*, *Bacillus* sp SM1A2; *Dictyoglomus thermophilum*, *Paenibacillus barcinonensis*, *Thermotoga maritema* MSB8 and *Acidothermus cellulolyticus* hydrolyze a wide range of substrates including beta-glucans, amorphous cellulose, and crystalline cellulosess [8-11].

Cellulase has various applications in starch processing, grain alcohol fermentation, in de-inking, in drainage improvement, malting and brewing. In paper industries cellulases are used to decrease the viscosity of the processed material during the pulping process and to improve sheet-strength properties of the end-product. Cellulase are extensively used in the bio-stoning of denim fabrics and production of environmentally friendly washing powders. In wine production cellulases are applied to obtain better fruit skin degradation, improved color extraction, easier must clarification, better extraction. *C. thermocellum* have been actively ferment cellulose and cellobiose to ethanol [12-13].

Considering the wealth of amazing biodiversity of thermophiles, the present study was undertaken to determine cellulase activity of thermophilic bacteria isolated from water and soil sediment of Tattapani Hot Spring. In present study novel thermophilic *Geobacillus sp* of Tattapani hotsprings were screened and characterized for extracellular thermophilic cellulase.

MATERIALS AND METHODS

Strains

The thermophilic bacterial strains used in this study were isolated from a water and sendiment of Tattapani hotspring, Himachal Pradesh, India.

Phylogenetic analysis showed that these isolates belong to genus Geo*bacillus*. The thermophilic bacterial isolates *Geobacillus kaustrophilus strain* PW11 (accession no. KF751758), *Geobacillus thermoleovorans* strain PW13 accession no. (KF751757), and *Geobacillus toebii* strain PS4 (accession no. KF751759) were characterized for extracellular thermophilic cellulase. The optimum temperature and pH for growth of these organisms was 80ºC and pH 7.0, respectively.

Characterization of cellulase from thermophilc bacterial isolates.

The thermophilic bacterial isolates (PW11, PW12, PW13 and PS4) were grown at 80°C and as a control DH5α at 37°C for 24 h with shaking at 200 rpm. Equal number of cells were spotted on LB agar medium supplemented in each plate with 1 % CMC to screen qualitatively for cellulase activity. As LB agar is not stable at 80°C, petri plates were incubated at 65°C for 24 hours. The CMC agar plate was flooded with Gram's iodine solution (1gm/100ml).

The appearance of clear zone around the bacterial growth indicated the utilization of CMC, thus indicative of cellulase activity. Quantitatively cellulase activity was measured by DNS method [14]. Cells were separated by centrifugation at 5000 rpm for 5 min at 4ºC. Total proteins present in the cell free spent medium were quantified by Bradford Method [15] and used as extracellular source of enzyme. Cellulase aactivity was measured by

the DNS method, through the determination of the amount of reducing sugars liberated from CMC.

Determination of the optimal pH, temperature, thermal stability and substrate for cellulase activity: Quantitatively, the optimal pH and temperature for cellulase activity of bacterial isolates was determined by using 10 µg proteins. Optimal temperature for enzyme activity was determined by incubating the reaction mixture at different temperatures ranging from 40-100°C.

Effect of pH on the activity of enzyme was studied by adjusting the pH of reaction buffer (5-11). Reaction buffers used include citrate-phosphate buffer (pH 5 and 6), phosphate buffer (pH 7) and Tris-HCl buffer (pH 8 and 9). Thermal stability of cellulase was determined by pre incubating protein (as a cell free spent medium) in phosphate buffer pH 7 at 100 °C. The cellulase activity was determined at intervals of 1 h.

To evaluate best substrate for cellulase activity, 1% CMC and filterpaper were used as substrate in quantitative assay. Quantitative enzyme assays were carried out as described [14].

Effect of chaotropic agents, metal ions and solvents on enzyme activity:

In order to check the effect of chaotropic agents, metal ions and solvents on cellulase activity, the extracellular enzyme was incubated with different concentrations (1, 5 and 10 mM) of protein inhibitors such as phenyl methyl sulfonyl fluoride (PMSF) and ethylene diamine tetraacetic acid (EDTA)]; salts of metal ions (Ni^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+} and Cd^{2+}) and detergents (0.5% and1%) such as sodium dodecyl sufate (SDS) and triton X-100. Various solvents such as ethanol, phenol, n-butanol, cyclohexane, hydrogen peroxide, pyridine and toluene (0.5% and1%) were assessed for effect on enzyme activities of thermophilic isolates.

The relative activity was calculated with respect to the control without adding inhibitors, metal ions and detergents.

RESULTS

Thermophilic bacterial isolates produce extracellular cellulase

To test the cellulase activity of the thermophilic bacterial isolates, the CMC agar plate having bacterial growth were flooded with Gram's iodine. After flooding starch agar plates with Gram's iodine, a varied size clear zone of diameter 29, 28, 29 and 30, mm was observed around the three bacterial isolate PW11, PW13 and PS4 respectively, thus indicated the presence of cellulase activity (Fig 1 A).

Pre incubated cellulase enzyme was thermostable at 100°C for 5 h

The cell free spent medium (supernatant) was used to assess optimal temperature and pH for cellulase activity. The enzyme exhibited cellulase activity at temperatures in the range of 50 – 100°C. The cellulase activity of bacterial isolate PW11 and PW12 was significantly increased with increase in temperature upto 80°C, with maximum activity of 2350 and 2721 U/mg respectively. Isolate PW13 and PS4 showed maximum cellulase activity 2571 and 2535 U/mg respectively at 90°C. Optimal pH for cellulase activity of bacterial isolate was determined in a buffer of pH values ranging from 5 to 9. The maximal cellulase activity of PW11, PW12, PW13 and PS4 was at pH 8, 6, 7 and 7 respectively. Drastic decrease (54 – 68 %) in the cellulase activity was observed at alkaline pH of 9 and 10 (Fig 1 B and C).

The pre incubated (100°C for 6 h) cell free spent medium was used to determine the thermostability of cellulase enzyme. The cellulase enzyme of bacterial isolate PW11, PW12, PW13 and PS4 was thermostable at 100°C for 5 h. After five hours, there was 20 – 32 % decrease in cellulase activity (Fig 1D).

Cellulase activity was determined by using substrates such as CMC and cellulose filter paper. Enzyme assay was carried out at 100°C for 30 min. Thermophilic bacterial isolates PW11 and PW13 exhibited 17 and 18 % decrease in cellulase activity respectively, when filter paper was used as substrate as compared to CMC. Bacterial isolate PW12 and PS4

exhibited 23 and 15 % enhanced cellulase activity respectively, when filter paper was used as substrate (Fig 1E.).

Metal, solvent and detergent tolerant cellulase activity

The cellulase activity of PW11 was enhanced by 2.8 and 2.5 folds in the presence of Mg^{2+} and Fe^{2+} respectively at 10 mM concentration. In the presence of metal ion such as Co^{2+}, Mn^{2+} Zn^{2+}, Ni^{2+}, Cu^{2+} (5mM), the celluase activity was enhanced by 2.7, 2.4, 2.2, 1.8 and 1.6 folds respectively. In contrast, Hg^{2+} at 1 mM concentration inhibited the cellulase activity by 23% (Fig 2 A).. The cellulase activity of PW12 was increased in the presence of metal ion such as Mn^{2+}, Co^{2+}, Fe^{2+}, Cd^{2+} by 4.82, 4.3, 2.7 and 2.35 folds respectively at 5 mM concentration. No change in activity was observed in the presence of metal ions such as Ca^{2+}, Mg^{2+}, Zn^{2+} and Hg^{2+} (Fig 2 B). In contrast, cellulase activity of PW13 was increased by 2.4, 2.6 and 2.4 folds in the presence of Fe^{2+} (10 mM), $Mg^{2+}$and Zn^{2+} (5 mM) respectively. 90 % and 72 % of original cellulase activity was retained in the presence of Cu^{2+} and Cd^{2+} (1 mM). Cellulase activity was completely inhibited in the presence of Hg^{2+} (Fig 2 C). The cellulase activity of PS4 was increased by 1.6 and 1.3 folds in the presence of concentration of Mn^{2+} and Ni^{2+} respectively at 10 mM concentration. No effect on cellulase activity was observed in the presence of all metal ions tested, except Hg^{2+} (Fig 2 D).

The cellulase activity of all the four isolate (PW11, PW12, PW13 and PS4) was stable in the presence of ethanol, n-butanol and cyclohexane. The cellulase activity of PW12 and PW13 was increased by 1.19 and 1.37 folds in the presence of 0.5 % ethanol, whereas isolate PW11 shows no significant increase in cellulase activity in the presence of 0.5 % ethanol. The enzyme activity of PW12 was not affected in the presence of phenol (1 %) and H_2O_2 (0.5 %). Enzyme activity of PS4 isolate was slightly enhanced by 1.1 folds in the presence of n-butanol and no change was observed in the presence of cyclohexane. The enzyme also retained 60 % activity in the presence of phenol, toluene and H_2O_2 at 0.5 % concentration (Fig 3 A - D).

The cellulase activity of all the four isolates was increased by 6 – 39 % in the presence of 0.1 % SDS and Triton X100. About 60 % enzyme activity was retained in the presence 0.5 % concentration of tested detergents (Fig 4 A - D). Bacterial isolate PW12, PW13 and PS4 showed increase in cellulase activity by 1.4, 1.6 and 1.3 folds in the presence of 1 mM EDTA respectively, while enzyme activity of PW11 was increased by 1.7 folds in the presence of .5 mM EDTA. There was 30 % inhibition of cellulase activity in the presence of 10 mM EDTA (Fig E - H).

DISSCUSSION

Maximum cellulase activity was exhibited by bacterial isolate of tattapani at temperature 80-90°C and pH 6-7. The cellulase enzyme produced by PW11, PW12, PW13 and PS4 was thermostable and exhibited activity even at 100°C. Thermophilic bacterial strain, *Geobacillus pallidus* having cellulase activity was isolated from Empty Fruit Bunch (EFB) and Palm Oil Mil Effluent (POME) compost [7]. A cellulase producing bacterium *Cellulomonas sp*. YJ5 was isolated from soil showed maximum cellulose activity at pH 7.0 and temperature 60°C [16]. The cellulase enzyme of *Anoxybacillus flavithermus* EHP2 showed a maximum activity between 65 - 75°C. The optimum temperature for cellulase produced by thermophilic *Bacillus* sp CH43 and *Bacillus* sp HR68 isolated from hot spring were 70°C and 65°C respectively [17] and showed reduction in the activity at 80°C. However, several archaebacteria showed optimum temperature range of 80-100°C cellulases activity [18].

The cellulase enzyme produced by PW11, PW12, PW13 and PS4 was unaffected in the presence of ethanol, n-butanol and cyclohexane. They also retained >60 % activity in the presence of phenol, toluene and H_2O_2.

Cellulase activity was enhanced by 1.2-1.5 folds in the presence of SDS (0.1 %), Triton X100 and 1 mM EDTA . The enzyme activity was completely inhibited by PMSF (1 mM). The cellulose activity of *Cellulomonas sp*. YJ5 was inhibited by Cu^{+2}, Fe^{+2}, Hg^{+2}, Cr^{+2} and SDS; whereas it was enhanced by cysteine and β-mercaptoethanol [16]

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Figure Legends

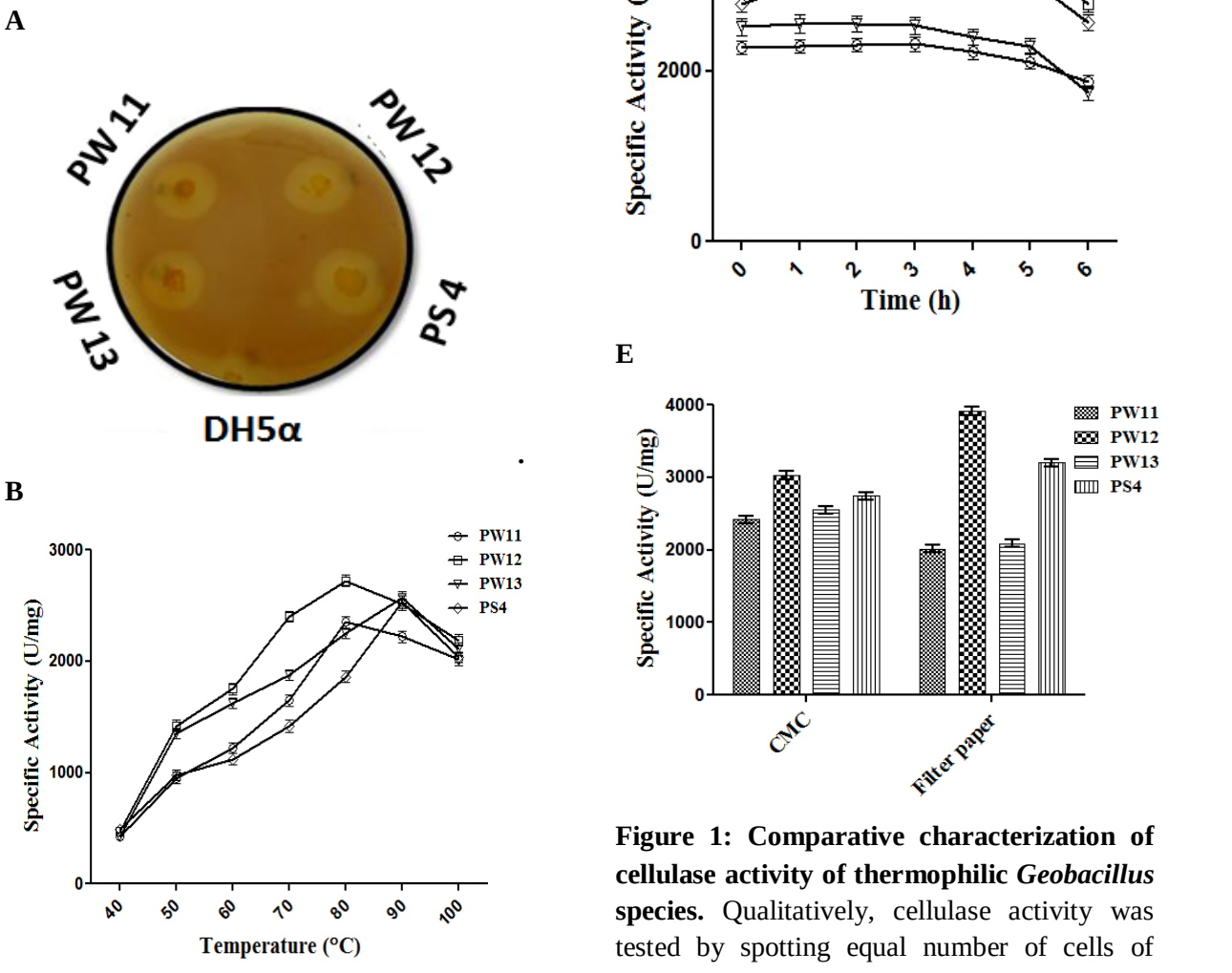

Figure 1: Comparative characterization of cellulase activity of thermophilic *Geobacillus* species. Qualitatively, cellulase activity was tested by spotting equal number of cells of thermophilic bacterial isolates and *E. coli* strain

(DH5α) on LB agar medium supplemented with 1% CMC. The plates were incubated at 65°C for 24 h and flooded with Gram's iodine and observed for the zone of clearance (**A).** Quantitatively, effect of temperature **(B),** and pH (**C**) on cellulase activity was studied by incubating the assay reactions at temperature ranging from 40-100 ^{O}C and pH 5-10. Thermostability of cellulase was performed by pre incubating the cell free spent medium at 100°C for different time intervals and measuring the cellulase activity at 100°C (**D**). Cellulase assays were performed in the presence of CMC and filter paper (**E**). Specific cellulase activity (U mg^{-1}) of three independent experiments was plotted with standard deviation as indicated. .

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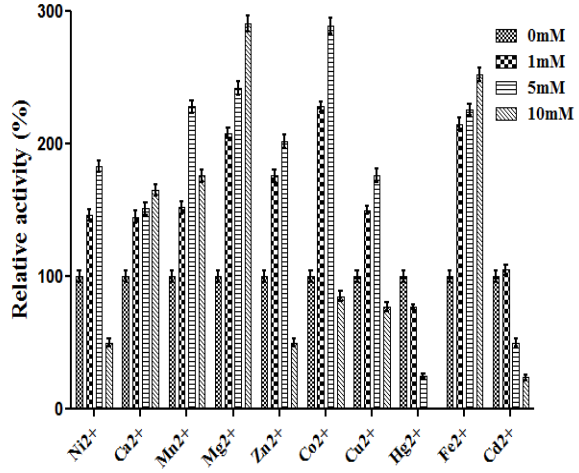

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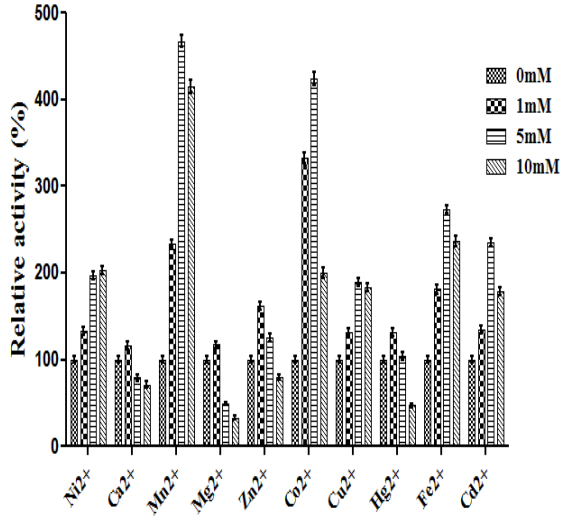

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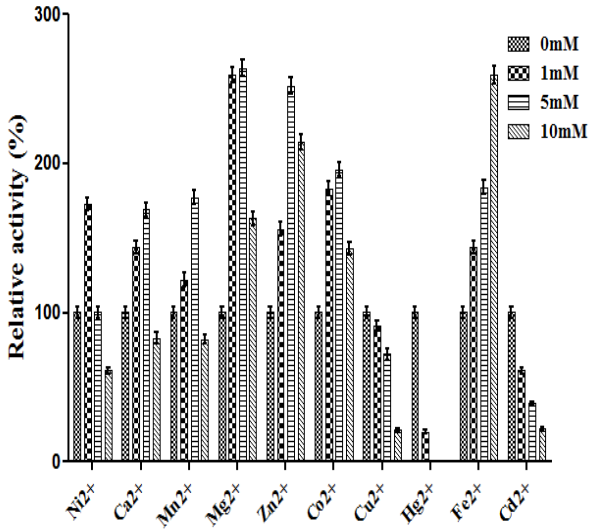

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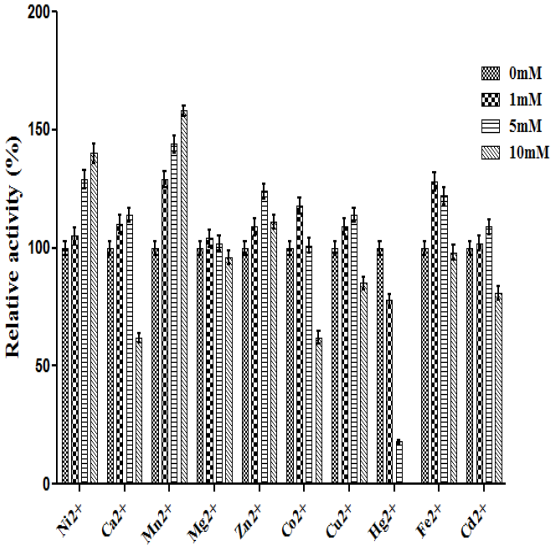
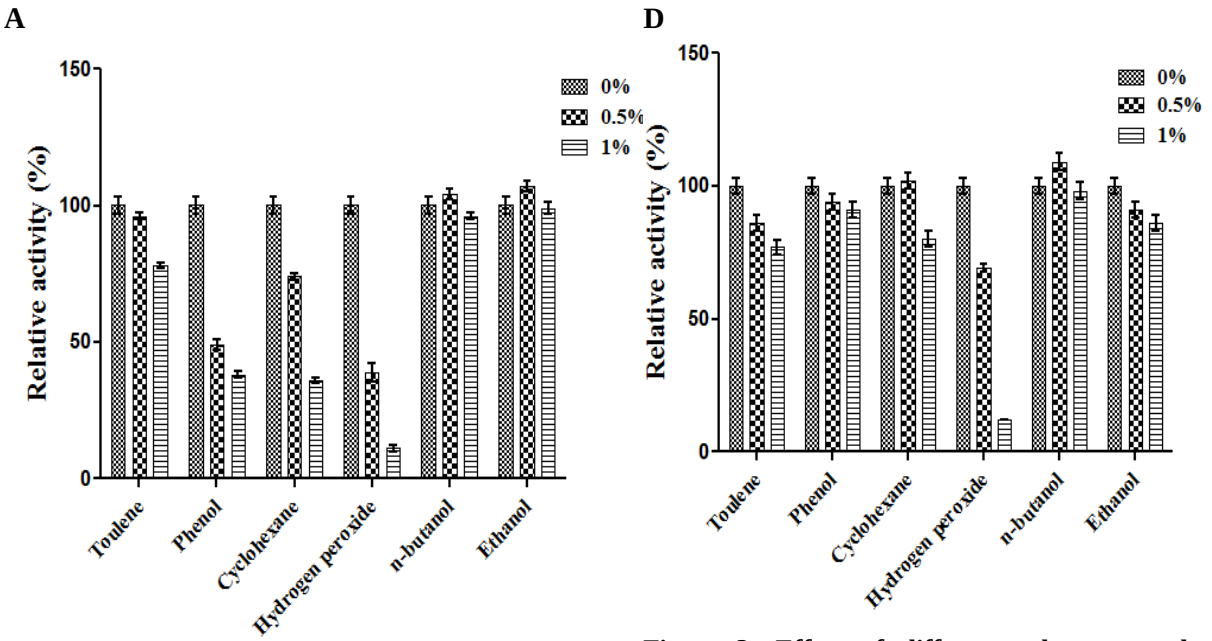
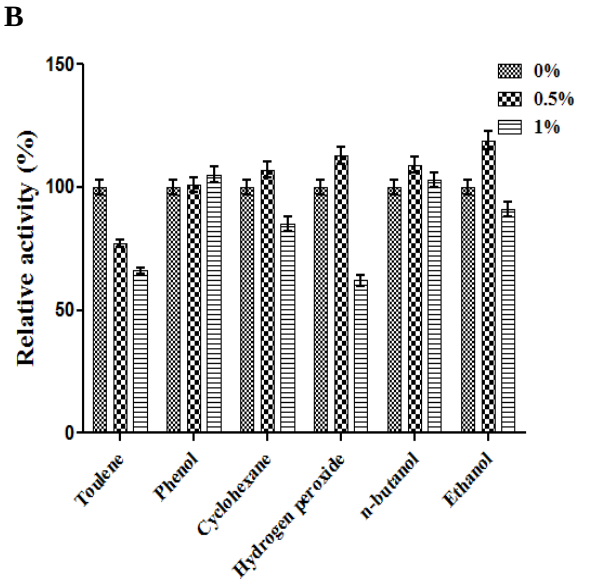


Figure 2: Effect of metal ions on the cellulase activity of thermophilic *Geobacillus* species. Reaction mixture was supplemented with salts of metal ions (Ni^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+}, Cd^{2+}) and enzyme assay was carried out at 90ºC, pH 7 for 30 min. The relative cellulase activity was plotted against the various metal ions and their concentration as indicated for PW11 **(A)**, PW12 (**B**), PW13(**C**) and PS4 (**D**). Reaction without supplementation of metal ions was served as control and considered as 100% cellulase activity. Data of three independent experiments was plotted with standard deviation.





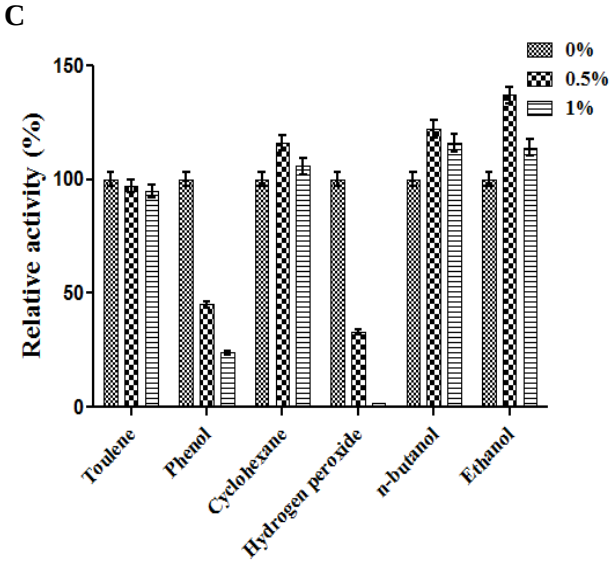
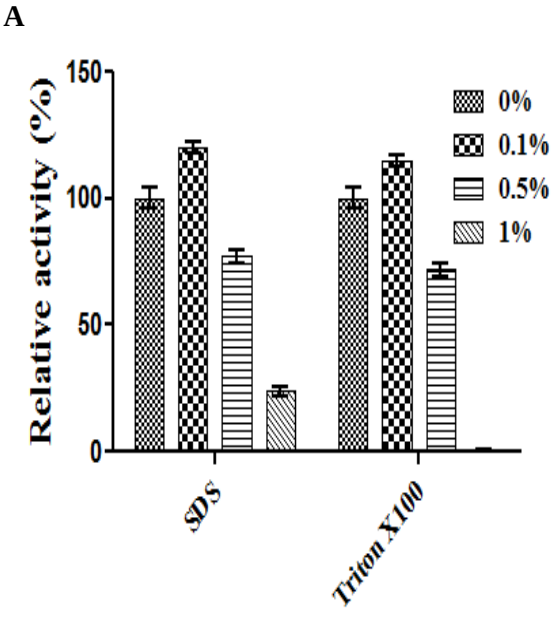


Figure 3: Effect of different solvents on the cellulase activity of thermophilic *Geobacillus* species.

Enzyme assays were carried out in the presence of different solvents at concentration of 0.5% and 1% and the relative cellulase activity of PW11 (**A**), PW12 (**B**), PW13(**C**) and PS4 (**D**) was plotted against the indicated concentration of solvents. Reaction without supplementation of solvents was served as control and considered as 100 % activity. Data of three independent experiments was plotted with standard deviation.



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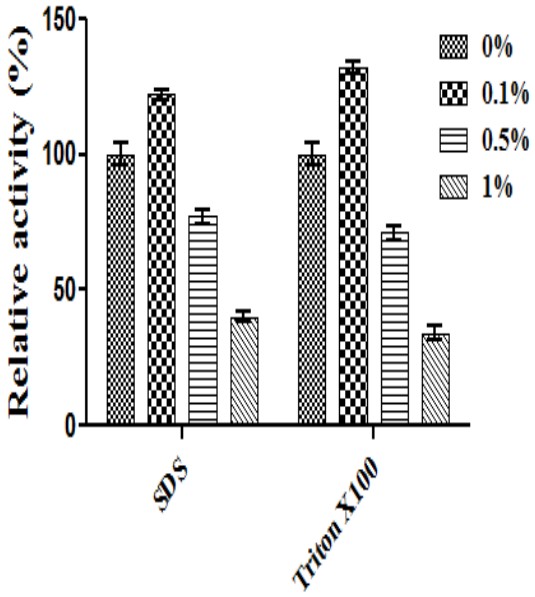
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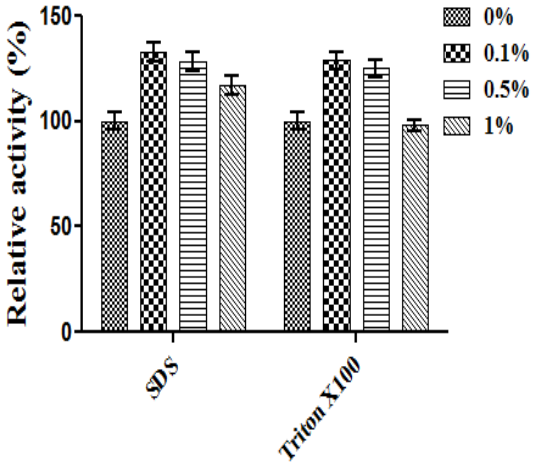
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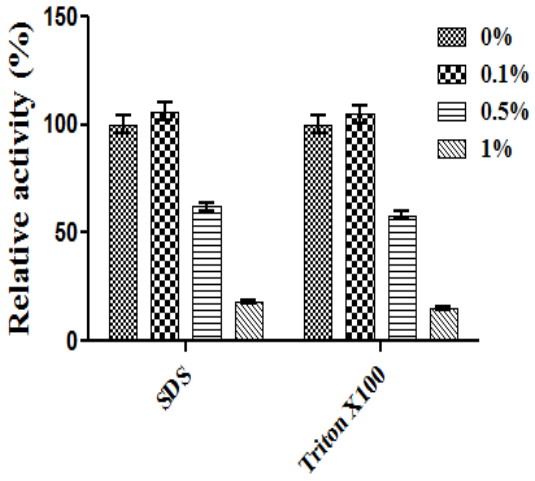
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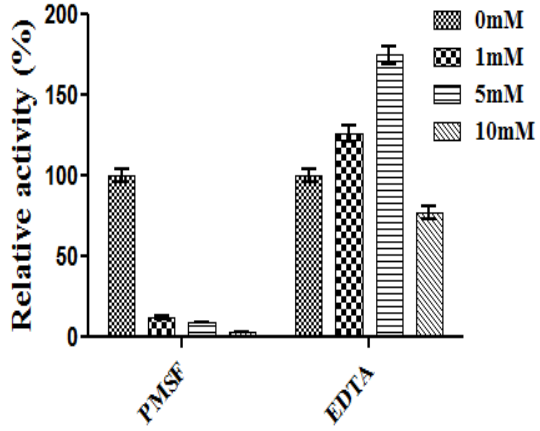
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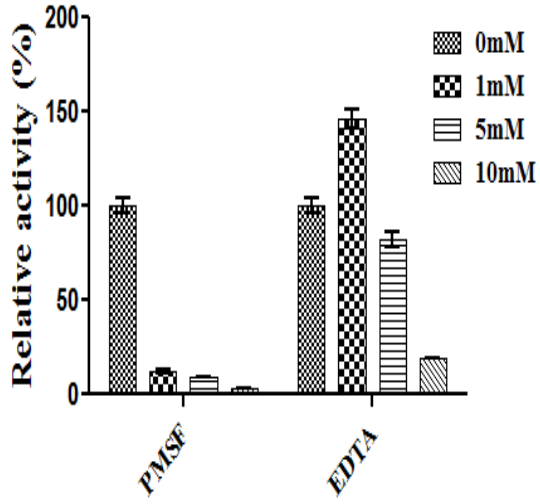
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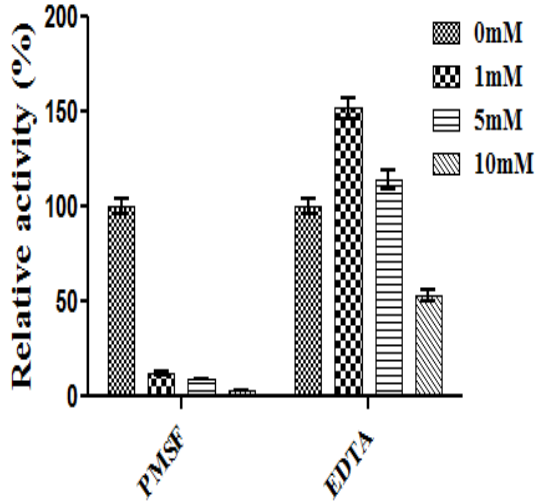
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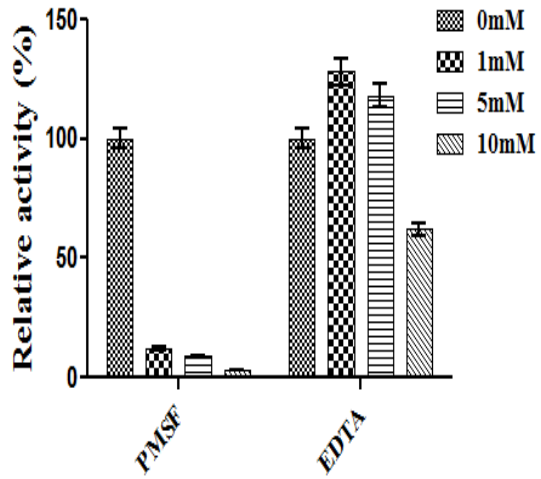

Figure 4: Effect of chaotropic agents on cellulase activity *Geobacillus* species. The relative cellulase activity of PW11 (A and E), PW12 (B and F), PW13 (C and G) and PS4 (D and H) was tested in the presence of indicated concentration of ionic (SDS) and non ionic (Triton X-100), EDTA and PMSF. Reaction without supplementation served as control and set as 100% activity. Data of three independent experiments was plotted with standard deviation.