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*Full Length Research Paper*

# Alkaline cellulase produced by a newly isolated thermophilic *Aneurinibacillus thermoaerophilus* WBS2 from hot spring, India

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**A thermophilic bacterium *Aneurinibacillus thermoaerophilus* WBS2 producing extracellular thermophilic cellulases was isolated from hot spring in India. Till date there is no report in literature about thermophilic cellulases produced by *A. thermoaerophilus* and optimization of nutritional factors for increasing cellulases yield. In order to enhance cellulase production, various fermentation parameters were optimized. Maximum cellulases production by *A. thermoaerophilus* WBS2 was obtained at pH 9.0 when growing in shake culture (150 rpm) at 65°C using 2% inoculum size. Using wheat and rice straw as substrates, 40 h of incubation period found to be optimum for exoglucanase activity whereas, for endoglucanase activity it was 60 h. Combination of inorganic and organic nitrogen source found to be most suitable. This is the first study to show that *A. thermoaerophilus* can degrade cellulose.**

**Keywords:** *Aneurinibacillus thermoaerophilus*, Thermophilic cellulases, hot spring, optimization of nutritional factors.

## INTRODUCTION

Cellulase enzyme hydrolyzes β-1,4-glycosidic bonds in cellulose polymer to release glucose units (Nishida et al., 2007). Complete enzymatic hydrolysis of cellulosic materials needs different types of cellulases viz. endoglucanases (CMCase), exoglucanases (FPase) and β-glucosidases (cellobiase) (Matsui et al., 2000). Major industrial applications of cellulases are in textile, detergents (Bhat, 2000), animal feed manufacturing (Dienes et al., 2004) and for biofuel generation through saccharification process (Da Silva et al., 2005). Cellulases are widely spread in nature, predominantly produced by microorganisms, (molds, fungi and bacteria) (Perez et al., 2002). Cellulolytic enzymes mostly produced from fungi *Trichoderma* and *Aspergillus* (Avendano and Cornejo, 1987). Since most industrial processes are carried out at high temperatures, therefore there is a great demand for thermophilic enzymes (Haki and Rakshit, 2003).

There has been increasing interest in cellulase production by bacteria because of fast growth rate (Petre et al., 1999). Cellulose degradation by thermophilic bacteria offers many advantages (Doi et al., 2003; Demain et al., 2005). Thermophilic bacterial cellulases have been reported from *Bacillus* sp. (Ray et al., 2007; Acharya and Chaudhary, 2011). Hot springs are natural habitat for thermophiles. Recently, thermophilic cellulose degrading bacteria have been isolated from Bakreshwar hot spring, West Bengal, India (Acharya and Chaudhary, 2011). *Aneurinibacillus thermoaerophilus* has recently been identified as a thermophilic lipase producer (Rahman et al., 2009; Masomian et al., 2010). However till now its potential about production of thermophilic cellulases has not been reported. In the present investigation, we hereby report about the thermophilic cellulose-degrading bacterium *A. thermoaerophilus*

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WBS2 and optimization of parameters for improving cellulase production by the cellulolytic isolate from Bakreshwar hot spring, India.

## MATERIALS AND METHODS

### Isolation and screening of cellulases producing thermophilic bacteria

Water samples, obtained from Bakreshwar hot spring (23°49′48″ N and 87°19′ 12″ E), West Bengal, India were collected aerobically in sterile plastic bottles (500 ml) and stored at -20°C. For enrichment of water samples for isolation of cellulases producing thermophilic bacteria, basic liquid media (BLM) (Patel et al., 2006) (g/L): $KH_2PO_4$: 1.36 g; $(NH_4)_2SO_4$: 1.0 g; $MgSO_4.7H_2O$: 0.2 g; $FeSO_4$: 10.0 mg; NaCl: 2.0 g; yeast extract, 1.0 g. supplemented with 0.3% carboxymethyl cellulose (CMC), was added to the water sample (1:1), mixed and incubated for 72 h at 55°C. The samples were then serially diluted in sterile distilled water, plated on agar medium consisting of BLM and plates were incubated at 55°C. After 24 h, morphologically distinct colonies were sub cultured on respective agar plates to get pure isolated colonies.

Screening for high cellulase producer was done by blood red cellulose stain (0.8% $I_2$ crystals, 0.8% KI, 8 mM KCl) (Ranoa et al., 2005) by point inoculating on above plates in duplicate. After 24 h incubation at 55°C, plates were flooded with aqueous red cellulose stain solution. Dye was drained after 10 to 15 min and plates were observed for production of halo zones surrounding the colonies. The isolates having colonies with high diffusible zones were screened as potential cellulases producer for further study. One particular isolate with high diffusible zone was purified by sub culturing on fresh plates. This isolate was preserved on the same agar medium at 4°C with periodic sub culturing.

### Analyses of 16S rDNA gene sequences

For identification of the selected isolate by 16S rDNA gene sequencing bacterial genomic DNA was extracted and purified (Minamisawa et al., 1992). 2 primers annealing at 5' and 3' end of the 16S rDNA were "AGAGTTTGATCMTGGCTCAG" and "TACGGYTACCTTGTTACGACTT" respectively. PCR amplification was performed in a final reaction volume of 100 µl. PCR reaction was run for 35 cycles in a DNA thermal cycler. The following thermal profile was used for PCR: denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min and extension at 72°C for 2 min. The final cycle included extension for 10 min at 72°C to ensure full extension of products. Amplified PCR products were then analyzed in agarose gel (1.0% w/v), excised from gel, and purified. 16S rDNA was sequenced and the 16S rDNA gene sequence of isolate was aligned with reference 16S rDNA sequences of GenBank using BLAST algorithm available in NCBI for identification of isolate which was found as *A. thermoaerophilus.* Partial 16S rDNA sequence of isolate was submitted to GenBank with accession number GU590783.

### Cellulase activity measurements

Organism was shake cultured (150 rpm) in BLM (Patel et al., 2006) supplemented with 0.3% CMC for 12 h at 55°C, and used to inoculate BLM. Cellulase activities were measured for cells grown in cellulolytic medium (100 ml) supplemented with either 0.3% CMC or 1% pretreated wheat and rice straw in Erlenmeyer flasks (250 ml) on an incubator cum shaker at 150 rpm. Rice and wheat straw were obtained from experimental field of Indian Agricultural Research Institute (IARI), dried at 50°C, ground to fine powder form to pass through 30 mesh sieve and pretreated with 4% NaOH overnight at room temperature and then washed and dried before use at 1% level as substrates for cellulase production. Aliquots from triplicate flasks were taken out and were centrifuged at 10,000 rpm for 10 min and cell free supernatants were used for different cellulolytic enzyme assays.

### Optimization of parameters for improving cellulase production

Most suitable pH of fermentation medium was determined by adjusting pH (6.0 to 10.0) at 1 unit interval using NaOH and HCl as and when required. For cellulase production by selected strains, fermentation was carried out between 50 to 75°C, up to 80 h, and production rate was measured at 20 h intervals. Fermentation medium was seeded with 1.0, 2.0, 3.0, 4.0 and 5.0% seed culture and incubated in shake culture (150 rpm). To detect appropriate nitrogen source for cellulase production by isolate, fermentation medium was supplemented with 2 inorganic (ammonium sulphate and sodium nitrate) and two organic (yeast extract and beef extract) nitrogen compounds at 0.2% level, thereby substituting prescribed nitrogen source of cellulolytic medium.

### Enzyme and protein assays

All enzyme assays were carried out in 50 mM sodium citrate buffer (pH 4.8). CMCase activity was determined (Ghose, 1987) with 1% solution of CMC as substrate. Release of reducing sugars in 30 min at 50°C was measured by DNSA method (Miller, 1959). FPase activity was assayed (Mandels et al., 1976) in a manner similar to that used to determine CMCase activity. A unit of activity was defined as the amount of enzyme required to liberate 1 µmol of glucose per min under assay conditions. Protein content of culture filtrates was also determined by folin-ciocalteu reagent (Lowry et al., 1951) using Bovine Serum Albumin (BSA) as standard.

### Statistical analysis

Data were statistically analyzed by Duncan's multiple range test at 0.05 probability level ($p<0.05$) using SPSS statistical software (SPSS for Windows, Release 12).

## RESULTS AND DISCUSSION

BLAST search of sequenced 16S rDNA of the isolate indicated that isolate was similar to *A. thermoaerophilus*. Survey of the literature revealed few reports on lipase produced by *A.* thermoaerophilus (Rahman et al., 2009; Masomian et al., 2010). But till date there is no report on cellulose degrading potential of *A. thermoaerophilus. A. thermoaerophilus* WBS2 isolated from hot spring in India has been shown to produce cellulases. Agricultural residues (wheat and rice straw) were used as substrates for optimization of extracellular cellulases production under various nutritional and environmental conditions.

### Effect of pH on cellulase production

The pH 9.0 was found to be optimal for CMCase and

**Table 1.** Effect of pH on cellulases production by *Aneurinibacillus thermoaerophilus* WBS2 using different substrates.

| pH | CMCase (IU/ml) | | | FPase (IU/ml) | | |
|---|---|---|---|---|---|---|
| | CMC | Wheat straw | Rice straw | CMC | Wheat straw | Rice straw |
| 6.0 | ND | 0.042±0.003$^{a}$ | 0.059±0.005$^{a}$ | 0.195±0.017$^{a}$ | 0.207±0.016$^{a}$ | 0.228±0.015$^{a}$ |
| 7.0 | 0.025±0.005$^{a}$ | 0.059±0.007$^{b}$ | 0.067±0.006$^{a}$ | 0.251±0.021$^{b}$ | 0.244±0.020$^{ab}$ | 0.290±0.017$^{c}$ |
| 8.0 | 0.080±0.006$^{b}$ | 0.060±0.008$^{b}$ | 0.080±0.007$^{b}$ | 0.415±0.035$^{d}$ | 0.362±0.031$^{c}$ | 0.331±0.021$^{d}$ |
| 9.0 | 0.080±0.007$^{b}$ | 0.058±0.004$^{b}$ | 0.081±0.011$^{b}$ | 0.403±0.023$^{d}$ | 0.426±0.033$^{d}$ | 0.319±0.025$^{cd}$ |
| 10.0 | 0.033±0.006$^{a}$ | 0.040±0.004$^{a}$ | 0.072±0.009$^{ab}$ | 0.352±0.020$^{c}$ | 0.257±0.019$^{b}$ | 0.261±0.022$^{ab}$ |

Mean values bearing the same superscript within a column didn't differ significantly (P>0.05) ND = Not detected; A unit of activity was defined as the amount of enzyme required to liberate 1µmol of glucose per minute under the assay conditions and expressed as IU/ml.

**Table 2.** Effect of temperature on cellulases production by *Aneurinibacillus thermoaerophilus* WBS2 using different substrates.

| Temperature (°C) | CMCase (IU/ml) | | | FPase (IU/ml) | | |
|---|---|---|---|---|---|---|
| | CMC | Wheat straw | Rice straw | CMC | Wheat straw | Rice straw |
| 50 | 0.022±0.003$^{a}$ | 0.020±0.005$^{a}$ | 0.038±0.005$^{b}$ | 0.305±0.022$^{c}$ | 0.313±0.018$^{b}$ | 0.225±0.020$^{b}$ |
| 55 | 0.056±0.005$^{b}$ | 0.048±0.005$^{b}$ | 0.065±0.008$^{c}$ | 0.326±0.021$^{cd}$ | 0.334±0.025$^{b}$ | 0.261±0.014$^{b}$ |
| 60 | 0.072±0.012$^{c}$ | 0.055±0.006$^{bc}$ | 0.070±0.008$^{c}$ | 0.358±0.032$^{d}$ | 0.352±0.024$^{b}$ | 0.331±0.021$^{c}$ |
| 65 | 0.080±0.007$^{c}$ | 0.060±0.008$^{c}$ | 0.081±0.011$^{c}$ | 0.415±0.035$^{e}$ | 0.426±0.033$^{c}$ | 0.319±0.021$^{c}$ |
| 70 | 0.049±0.006$^{b}$ | 0.044±0.008$^{b}$ | 0.033±0.004$^{b}$ | 0.184±0.018$^{b}$ | 0.242±0.020$^{a}$ | 0.172±0.021$^{a}$ |
| 75 | 0.032±0.005$^{a}$ | 0.023±0.004$^{a}$ | 0.015±0.004$^{a}$ | 0.105±0.012$^{a}$ | 0.211±0.015$^{a}$ | 0.124±0.015$^{a}$ |

Mean values bearing the same superscript within a column didn't differ significantly (P>0.05). A unit of activity was defined as the amount of enzyme required to liberate 1µmol of glucose per minute under the assay conditions and expressed as IU/ml.

FPase production using wheat straw (Table 1). But FPase production using CMC and rice straw as substrate, pH 8.0 was found to be ideal but not significantly differ from pH 9.0 (P>0.05). *A. thermoaerophilus* WBS2 was identified as alkalophilic in nature and there was insufficient cellulase yield was observed at acidic to neutral range of pH. The pH 8.0 was also found to be optimal for FPase activity by a newly isolated thermophilic *Brevibacillus* sp. strain JXL (Liang et al., 2009) but FPase activity (0.02 IU/ml) reported from *Brevibacillus* sp. strain JXL was much lesser than FPase (0.426 IU/ml using wheat straw) produced by *A. thermoaerophilus* WBS2 in present experiment (Table 1). Kim et al. (2005) also identified and characterized alkaline cellulase produced by a newly *Bacillus* sp. HSH-810. But Souichiro et al. (2004) reported the optimum initial pH for growth and cellulose degradation of *Clostridium straminisolvens* sp. nov. at 7.5. In contrast, production of acidic cellulase has also been reported (Hagerdal et al., 1979) and pH 7.0 was found optimum for the production of endoglucanase (CMCase) by *Cellulomonas* and *Micrococcus* sp. (Immanuel et al., 2006). Alkaliphilic properties of cellulases produced by *Bacillus* sp. have also been studied, due to the possible application of these enzymes in detergent industry (Ozawa et al., 2001; Acharya and Chaudhary, 2011).

**Effect of temperature on cellulase production**

Among three substrates used (CMC, Wheat and rice straw) in study, higher FPase activity (0.426 IU/ml) was obtained on wheat straw at 65°C (Table 2). Using rice straw as substrate, FPase (0.331 IU/ml) showed maximum activities at 60°C which did not differ significantly from 65°C (P>0.05) (Table 2). Acharya and Chaudhary (2011) also reported similar kinds of results for *Bacillus licheniformis* and *Bacillus* sp. Higher cellulolytic activities (FPase and cellobiase) were obtained from *Trichoderma reesei* GM9414 on wheat straw as carbon source (Gonzalez et al., 1986). Souichiro et al. (2004) reported optimum temperature for growth and cellulose degradation of *C. straminisolvens* sp. nov. at 50 to 55°C.

**Effect of incubation period on cellulases production**

Effect of incubation time on enzyme production was studied from 8 to 40 h (Table 3a) and 20 to 80 h (Table 3b) using CMC and rice and wheat straw as substrate respectively. Higher production of both CMCase (0.080 IU/ml) and FPase (0.415 IU/ml) by *A. thermoaerophilus* WBS2 were obtained after 24 h of incubation period and after 40 h 47% decrease in FPase yield was observed

**Table 3a.** Effect of incubation period on cellulases production by *Aneurinibacillus thermoaerophilus* WBS2.

| Substrate (CMC) | Incubation time (h) | | | | | |
|---|---|---|---|---|---|---|
| **Cellulase activity (IU/ml)** | **8** | **16** | **24** | **32** | **40** | **48** |
| CMCase | 0.051±0.005$^{a}$ | 0.067±0.007$^{b}$ | 0.080±0.007$^{d}$ | 0.075±0.008$^{cd}$ | 0.072±0.005$^{c}$ | 0.044±0.005$^{a}$ |
| FPase | 0.117±0.016$^{a}$ | 0.398±0.034$^{d}$ | 0.415±0.035$^{d}$ | 0.231±0.024$^{c}$ | 0.221±0.023$^{c}$ | 0.180±0.018$^{b}$ |
| Protein | 125±6.5$^{a}$ | 250±5.5$^{e}$ | 240±7.0$^{e}$ | 210±7.5$^{d}$ | 170±5.5$^{c}$ | 145±6.0$^{b}$ |

Mean values bearing the same superscript within a row didn't differ significantly (P>0.05). ND* = Not detected; A unit of activity was defined as the amount of enzyme required to liberate 1 µmol of glucose per minute under the assay conditions and expressed as IU/ml.

**Table 3b.** Effect of incubation period on cellulases production by *Aneurinibacillus thermoaerophilus* WBS2 using wheat and rice straw as substrate.

| Incubation time (h) | CMCase (IU/ml) | | FPase (IU/ml) | |
|---|---|---|---|---|
| | **Wheat straw** | **Rice straw** | **Wheat straw** | **Rice straw** |
| 20 | 0.049±0.006$^{a}$ | 0.034±0.005$^{a}$ | 0.280±0.025$^{b}$ | 0.204±0.019$^{a}$ |
| 40 | 0.055±0.008$^{ab}$ | 0.044±0.006$^{a}$ | 0.426±0.033$^{c}$ | 0.331±0.021$^{b}$ |
| 60 | 0.060±0.008$^{b}$ | 0.081±0.011$^{c}$ | 0.398±0.031$^{c}$ | 0.311±0.015$^{b}$ |
| 80 | 0.044±0.005$^{a}$ | 0.053±0.006$^{b}$ | 0.218±0.020$^{a}$ | 0.182±0.019$^{a}$ |

Mean values bearing the same superscript within a column didn't differ significantly (P>0.05). A unit of activity was defined as the amount of enzyme required to liberate 1µmol of glucose per minute under the assay conditions and expressed as IU/ml.

**Table 4.** Effect of inoculum size on cellulases production by *Aneurinibacillus thermoaerophilus* WBS2 using different substrates.

| Inoculum size (%) | CMCase (IU/ml) | | | FPase (IU/ml) | | |
|---|---|---|---|---|---|---|
| | **CMC** | **Wheat straw** | **Rice straw** | **CMC** | **Wheat straw** | **Rice straw** |
| 1.0 | 0.070±0.007$^{ab}$ | 0.059±0.007$^{b}$ | 0.080±0.009$^{b}$ | 0.403±0.016$^{ab}$ | 0.417±0.033$^{c}$ | 0.310±0.016$^{a}$ |
| 2.0 | 0.080±0.007$^{c}$ | 0.060±0.008$^{b}$ | 0.081±0.011$^{b}$ | 0.415±0.035$^{c}$ | 0.426±0.033$^{c}$ | 0.317±0.018$^{ab}$ |
| 3.0 | 0.080±0.012$^{c}$ | 0.058±0.007$^{b}$ | 0.072±0.010$^{ab}$ | 0.375±0.029$^{ab}$ | 0.350±0.021$^{ab}$ | 0.331±0.021$^{b}$ |
| 4.0 | 0.074±0.010$^{b}$ | 0.045±0.006$^{a}$ | 0.065±0.009$^{a}$ | 0.355±0.027$^{a}$ | 0.345±0.024$^{ab}$ | 0.315±0.025$^{ab}$ |
| 5.0 | 0.060±0.006$^{a}$ | 0.040±0.005$^{a}$ | 0.062±0.008$^{a}$ | 0.352±0.023$^{a}$ | 0.325±0.020$^{a}$ | 0.291±0.016$^{a}$ |

Mean values bearing the same superscript within a column didn't differ significantly (P>0.05). A unit of activity was defined as the amount of enzyme required to liberate 1µmol of glucose per minute under the assay conditions and expressed as IU/ml.

(Table 3a). At the same incubation period (24 h) Ariffin et al. (2006) recorded much lesser FPase (0.011 IU/ml) and CMCase (0.079IU/ml) activities by *Bacillus pumilus* EB3. Soluble protein content increased during cultivation period and reached maximum (250µg/ml) after 16 h of incubation and then decreased by end of cultivation (Table 3a). Thus increase in activity of enzymes is associated with an increase in extracellular protein levels.

When wheat and rice straw were used as substrates, it was observed that higher FPase production occurred after 40 h of incubation period whereas for CMCase production, 60 h (Table 3b). Peak CMCase activity of *Aspergillus niger* and *Aspergillus terreus* was observed on the 8$^{th}$ day of fermentation (0.12 and 0.10 IU/ml respectively) growing on cassava waste (Pothiraj et al., 2006). Pothiraj et al. (2006) also recorded maximum FPase activity (0.46 IU/ml) after 10 days of incubation period by *Rhizopus stolonifer* on cassava waste. Results indicated maximum production of both CMCase and FPase at relatively much shorter period of time by *A.thermoaerophilus* WBS2 over other strains and suggested usefulness for enzyme production.

## Effect of inoculum size on cellulases production

For optimum enzyme production in fermentation medium, inoculum size was optimized. 2% inoculum size was found to be better for maximum enzyme production (Table 4). However, enzyme production by the strain in 3% inoculum size was not significantly different (P>0.05) from that in 2% inoculum size (Table 4). For production of raw starch hydrolyzing amylase by *Bacillus*, 2% inoculum was recommended (Avendano and Cornejo, 1987).

**Table 5.** Effect of various nitrogen sources on cellulases production by *Aneurinibacillus thermoaerophilus* WBS2 using different substrates.

| Nitrogen source | CMCase (IU/ml) | | | FPase (IU/ml) | | |
|---|---|---|---|---|---|---|
| | CMC | Wheat straw | Rice straw | CMC | Wheat straw | Rice straw |
| Control | 0.080±0.007$^{b}$ | 0.060±0.008$^{c}$ | 0.081±0.011$^{c}$ | 0.415±0.035$^{a}$ | 0.426±0.033$^{c}$ | 0.331±0.021$^{c}$ |
| $(NH4)_2SO_4$ | ND | 0.060±0.008$^{c}$ | 0.080±0.010$^{d}$ | ND | 0.277±0.019$^{b}$ | 0.207±0.014$^{a}$ |
| $NaNO_3$ | ND | 0.071±0.009$^{c}$ | 0.048±0.006$^{b}$ | ND | 0.235±0.015$^{a}$ | 0.240±0.017$^{ab}$ |
| Beef extract | 0.082±0.012$^{b}$ | 0.028±0.005$^{a}$ | 0.029±0.004$^{a}$ | 0.431±0.026$^{ab}$ | 0.283±0.020$^{b}$ | 0.268±0.016$^{b}$ |
| Yeast extract | 0.065±0.009$^{a}$ | 0.044±0.006$^{b}$ | 0.053±0.005$^{bc}$ | 0.456±0.038$^{b}$ | 0.206±0.021$^{a}$ | 0.310±0.028$^{c}$ |

Mean values bearing the same superscript within a column didn't differ significantly (P>0.05). ND = Not detected; A unit of activity was defined as the amount of enzyme required to liberate 1µmol of glucose per minute under the assay conditions and expressed as IU/ml.

Inoculum size (2-3%) was also found to be optimum for cellulases produced by *Bacillus subtilis* CY5 and *Bacillus circulans* TP3 (Ray et al., 2007).

## Effect of various nitrogen sources on cellulases production

Effect of various nitrogen sources on cellulase production by isolate *A. thermoaerophilus* WBS2 is shown in Table 5. Results revealed that combination of inorganic and organic source of nitrogen (served as control) was the most effective for both CMCase and FPase production on all three substrates. Present results showed lower cellulase activity with inorganic nitrogen apparently suggesting reduced utilization of inorganic nitrogen by *A. thermoaerophilus* WBS2. Contrary to that, Spiridonov and Wilson (1998) found $NH_4$ compounds are most favourable nitrogen sources for protein and cellulase synthesis by *Thermomonospora fusca*. Rajoka(2004) reported $KNO_3$ and $NH_4NO_3$ as the best nitrogen sources for cellulase synthesis in *Cellulomonas flavigena*.

## Conclusions

In summary, *A. thermoaerophilus* WBS2 is a potentially thermophilic cellulolytic bacterium isolated from an Indian hot spring. This is the first report on cellulose degrading ability of *A. thermoaerophilus*. The results add significant knowledge on diversity of thermophilic cellulolytic bacteria and possible exploration of the thermophile for the production of industrial enzyme.

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