



## Characterization of industrially important cellulase produced by *Actinomyces bovis* isolated from landfill site

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### ABSTRACT

Cellulose is homo-polymer of glucose unit. It is one of the causes of environmental pollution and creates various health hazards if not decomposed properly. In the present work, *Actinomyces bovis* was isolated from enriched sample collected from landfill site located at Chakur, Dist. Latur (India). It was produced 520 U/mL of cellulase under submerged state fermentation. The dialyzed *Actinomyces* cellulase showed optimum activity at temperature 30 °C and pH 6.0. The enzyme retained 10-35 % of its initial activity in the presence of metal ions ( $\text{Sn}^{++}$ ,  $\text{Hg}^{++}$ ,  $\text{Co}^{++}$ ,  $\text{Cu}^{++}$ ,  $\text{Ca}^{++}$  and  $\text{Zn}^{++}$  at 1 mM concentration) and surfactant (at 0.1% concentration). The enzyme was also stable in the presence of commercial laundry detergents at 7 mg/mL concentration. It was retained about 40-60 % of its initial activity in the presence of Ariel<sup>TM</sup>, Wheel<sup>TM</sup>, Ghari<sup>TM</sup>, Surf Excel<sup>TM</sup>, Tide<sup>TM</sup> and Nirma<sup>TM</sup>. Hence, the cellulase isolated from *Actinomyces bovis* may find application in various bioprocesses and in bioconversion of biomass.

**Keywords:** Cellulose, Biodegradation of biomass, Catalyst, SmF

### INTRODUCTION

Cellulose is a linear disaccharide glucose unit with 1, 4-  $\beta$ - D glycoside linkages. It is versatile and most abundant organic component in plants found on earth. It has received much attention as substrate for production of biomolecules and biofuel. Many microorganisms have reported with cellulase activities including bacteria and fungi which can utilize it effectively as a substrate. Cellulytic activity is a basically biologically controlled process by the enzymes involved in cellulose system. Cellulase enzymes system encompasses three classes of soluble extracellular enzymes such as 1, 4-  $\beta$ -endoglucanase, 1, 4-  $\beta$ -exoglucanase and  $\beta$ - glycosidase. Microorganisms including bacteria and fungi having ability to produce and degrade cellulosic compounds have received primary importance in various industries and bioprocesses. *Chaetomium*, *Fusarium*, *Myrothecium*, *Trichoderma*, *Penicillium*, *Aspergillus*, and many other fungal species have been reported and responsible for hydrolysis of cellulosic biomass. Cellulytic *Actinomyces*, *Bacilli*, *Bacteroides*, *Butyrivibrio*, *Clostridium*, *Cellulomonas*, *Methanobrevibacter*, *Pseudomonads*, *Trichonympha* and *Ruminiococcus albus*, etc. produce cellulase enzyme with high enzymatic activity as compared to other genera [1-5].

Cellulase due to its massive application such been used in various processes such as enzymatic clarification, production of biogas, chiral separation triphasic ligand binding studies agricultural and plant waste management, starch processing, malting and brewing, whitening of pulp, clearing of vegetables juices, and textile industry. Huge amount of cellulosic waste generated on daily basis from various life processes ranged from agricultural to domestic usage [6-7]. The generated cellulosic waste is usually collected and by municipal organizations and disposed of in open field or incinerated. These traditional practices are one of the causes for emission of green house gases like carbon dioxide and methane, polychlorinated P-dioxins and diazofurans, which creates health problems such as risks of bladder, brain and cancers in children. The developing countries like India, China, Pakistan, Bangladesh, Nepal etc. suffering from problem of cellulosic solid waste management [8-10].

In present paper, cellulase producer was isolated from landfill site and produced industrially importance cellulase was characterized.

## EXPERIMENTAL SECTION

### Sample collection

The composite soil sample was collected in sterile polypropylene bags from dumping sites in Chakur, Dist. Latur (India).

### Enrichment of culture media

The 1gm of fine collected soil sample was weighed and transferred to MSM medium supplemented with slurry of 1% different cellulosic substrate such as Carboxy methyl cellulose (CMC), filter paper, drawing paper separately. All flasks were kept on shaking incubator 120 rpm for 7 day at temperature 30 °C. After the incubation, the cellulase producer was isolated [11].

### Isolation cellulase producer from enriched medium

The 0.1 ml enriched medium was spread on MSM agar plate containing 1% different cellulosic substrate i.e. CMC, filter paper, drawing paper separately. The Petri plates were incubated for 7 day at 30 °C. After completion of incubation, the plates were flooded 0.1% Congo red dye (for 15 min). Then, the plates were washed with 1.0 M sodium chloride solution for 20min and observed for clearance zone. Presence of zone of clearance indicate positive test [12].

### Morphological and biochemical characterization

The microorganisms showing positive cellulase tests were selected and colony characteristics were recorded. The selected isolates were obtained as pure culture. All the pure cultured isolated were tested for their biochemical characteristics such as Gram staining, motility, utilization of carbohydrates and IMViC. The enzyme producing ability was determined using qualitative tests [13].

### Production, extraction and partial purification of cellulase

The production of cellulase was carried out using submerged state fermentation method. The MSM medium was prepared in phosphate buffer (pH 6.8) and supplemented with 1% CMC as carbon source. The autoclaved production medium was inoculated with 5% of inoculum of selected isolate. The inoculated medium was incubated at 30 °C in shaking incubator at 120 rpm speed for 10 days. After completion of incubation period, the production medium was centrifuged at 15000 rpm at 4 °C temperature. The filtrate was collected and produced cellulase was precipitated at 65% ammonium sulphate saturation. The content was kept at 4 °C overnight to precipitate residual dissolved enzyme. On next day, the precipitated enzyme was separated using centrifugation technique at 1000 rpm at 4 °C temperature. The precipitated enzyme was suspended in 5 mL citrate buffer (pH 5.8, 0.1 M) and poured in dialysis bag (30 kDa pore size). The clamped dialysis bags containing cellulase enzyme was dialyzed against same buffer (0.005 M) first for the two hours. Then buffer was changed. Again this content was dialyzed for two hours and buffer was changed. In a freshly prepared buffer the clamped dialysis bags were dialyzed overnight. This dialyzed and partially purified enzyme was stored at 4 °C temperature until use [14-15].

### Enzyme Assay

Total cellulase activity was determined by measuring the amount of reducing sugar formed from CMC. The enzyme activity was determined according to the methods recommended by the International Union of Pure and Applied Chemistry (IUPAC) Commission on Biotechnology. Activity was determined by incubating 0.5 ml of purified enzyme with 0.5 mL CMC (0.5 %) prepared in sodium phosphate buffer (pH 6.0) at 30 °C for 30 min. After incubation, the reaction was terminated by addition of 3 mL of 3, 5- di-nitro-salicylic acid (DNSA) reagent. The content was placed in boiling water bath for 10 min. to develop colored complex. The content was cooled at room temperature (RT). The 5 mL distilled water was added and vortex. The optical density was taken at 540 nm using calorimeter and recorded [16].

### Effect on temperature on activity and stability of cellulase

The dialyzed cellulase was incubated at temperature ranged from 10 to 80 °C for 30 min. The cellulase + 0.5 % substrate + sodium phosphate buffer (pH 6.0) was mixed as 1:1:1 ratio and incubated at respective temperature for 10 min. After incubation, the reaction was terminated by addition of 3 mL of DNSA reagent. The content was placed in boiling water bath for 10 min. to develop colored complex. The content was cooled at RT. The 5 mL distilled water was added and vortex. The optical density was taken at 540 nm using calorimeter and recorded.

**Effect of pH on activity and stability of cellulase**

Different buffers having pH 3 to 11 were prepared. The cellulase + 0.5 % substrate + buffer were added as 1:1:1 ratio and incubated at 30 °C temperature for 10 min. After incubation, the reaction was terminated by addition of 3 mL of DNSA reagent. The content was placed in boiling water bath for 10 min. to develop colored complex. The content was cooled at RT. The 5 mL distilled water was added and vortex. The optical density was taken at 540 nm using calorimeter and recorded.

**Effect of different metal ion on activity and stability of cellulase**

The effect of different metal ions (CuCl<sub>2</sub>, ZnCl<sub>2</sub>, SnCl<sub>2</sub>, HgCl<sub>2</sub>, CaCl<sub>2</sub> and CoCl<sub>2</sub> at 1 M concentration) on the activity and stability of cellulase was studied. The metal ion solution + 0.5 % substrate + buffer were taken in 1:1:1 ratio and incubated at temperature 30 °C and pH 6.0 for 10 min. After incubation, the reaction was terminated by addition of 3 mL of DNSA reagent. The content was placed in boiling water bath for 10 min. to develop colored complex. The content was cooled at RT. The 5 mL distilled water was added and vortex. The optical density was taken at 540 nm using calorimeter and recorded.

**Effect of surfactant Sodium didecyl sulphate (SDS) on activity and stability of cellulase**

The effect of SDS on the activity stability was studied by preparing the different concentrations such as 0.1 %, 0.2 %, 0.5 %, 0.8 % and 1 %. SDS + 0.5 % substrate + buffer were taken in 1:1:1 ratio and incubated at temperature 30 °C and pH 6.0 for 10 min. After incubation, the reaction was terminated by addition of 3 mL of DNSA reagent. The content was placed in boiling water bath for 10 min. to develop colored complex. The content was cooled at RT. The 5 mL distilled water was added and vortex. The optical density was taken at 540 nm using calorimeter and recorded.

**Effect of commercially used laundry detergent on activity and stability of cellulase**

Laundry detergents such as Ariel<sup>TM</sup>, Ghari<sup>TM</sup>, Surf Excel<sup>TM</sup>, Wheel<sup>TM</sup>, Tide<sup>TM</sup> and Nirma<sup>TM</sup> were dissolved in distilled water at concentration 7 mg/mL. 10 mL prepared quantity have detergent at 7mg/mL concentration. The content was boiled for 10 min to deactivate the enzyme present in the detergent and cooled at RT. The cooled detergent solution was filtered through Whatman filter paper no.1 and collected. The filtered content was making up to 10 mL to maintain its concentration. 7mg/mL. The detergent + 0.5 % substrate + buffer were taken in 1:1:1 ratio and incubated at temperature 30 °C and pH 6.0 for 10 min. After incubation, the reaction was terminated by addition of 3 mL of DNSA reagent. The content was placed in boiling water bath for 10 min. to develop colored complex. The content was cooled at RT. The 5 mL distilled water was added and vortex. The optical density was taken at 540 nm using calorimeter and recorded [17-18].

**Statistical analysis**

The entire tests performed in triplication and standard deviation for individual test result was calculated.

**RESULTS AND DISCUSSION**

The colony count for enriched MSM medium was  $3 \times 10^2$  CFU/mL. From the enriched medium, 15 isolates were isolated. Of these six isolates were showed positive cellulase test. These are named as LFC1 to LFC6. Of the six isolates, LFC4 showed maximum production cellulase detected using CMC as carbon source in a qualitative test. Therefore, it was chosen for further study and production for cellulase. Colony of LFC4 was white, 2 mm, circular, entire, flat, opaque and non-sticky. The cells were very long Gram positive rod motile rod shaped. It showed optimum temperature 30 °C and pH 6.0. It was utilized glucose, fructose and lactose as carbon source. It was showed positive tests for amylase, urease, indole production, MR, citrate utilization and H<sub>2</sub>S production (Table 1). According Bergey's Manual of Systematic Bacteriology (1994) [19], cellulase producer LFC4 was identified as *Actinomyces bovis* (*A. bovis*).

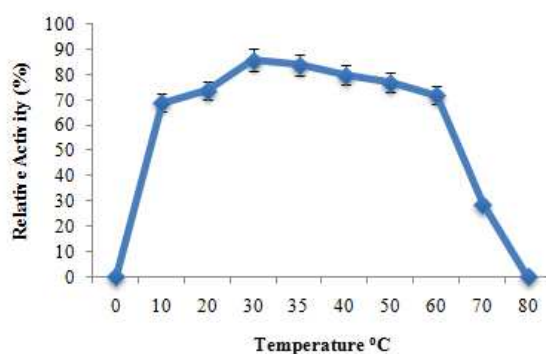
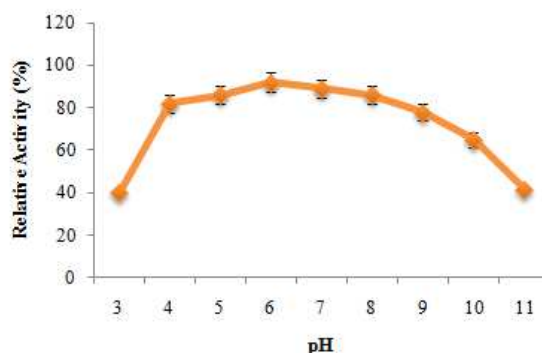
**Production partial purification and characterization of cellulase produced by *A. bovis* under submerged state fermentation**

The isolated *A. bovis* produced produced 520 U/mL of cellulase at temperature 30 °C and pH 6.0. Ghose (1987) and Goldbeck et al., (2013) observed similar type of results. They have recorded that *Actinomyces* produced cellulase showed the remarkable activity at temperature 30 °C and at pH 6.0 (Fig. 1-2) [20-21].

Hence, temperature 30 °C and at pH 6.0 were chosen as optimal parameter to carry out further experiments. Those parameter optimized were adopted and used in further experiments/tests.

Table 1. Morphological and biochemical characteristics of *A. bovis*

Parameter	Result
Cell shape	Very long rod
Gram staining	+
Motility	Non-motile
Color of colony	White
Size of colony (mm)	2
Shape of colony	Circular
Margin of colony	Entire
Elevation of colony	Flat
Opacity	Opaque
Consistency	Non-sticky
Optimum temperature (°C)	30
Optimum pH	6.0
Glucose	+
Lactose	+
Fructose	+
Starch	+
Cellulase	+
Urease	+
Indole production	+
Methyl red	+
Voges-Proskauer	-
Citrate utilization	+
H <sub>2</sub> S Production	+

Fig. 1. Effect of temperature on activity of cellulase produced by *A. bovis*Fig. 2. Effect of pH on activity of cellulase produced by *A. bovis*

*Actinomyces* cellulase showed maximum activity and stability at varied ranges of temperature and pH range from 10 to 70 °C and pH 4-10 respectively. Similar types of result reported worldwide by various research groups. Pirzadah et al., (2010) characterized *Actinomyces* and *Trichoderma* cellulase. They have reported that cellulase produced by these species were stable at varied range of temperatures and pH 50 to 70 °C and pH 4.8 to 5.5 respectively [22].

*Actinomyces* cellulase also showed remarkable activity in the presence of SnCl<sub>2</sub> followed by CuCl<sub>2</sub> and HgCl<sub>2</sub> followed by ZnCl<sub>2</sub> and CaCl<sub>2</sub>. The *Actinomyces* cellulase showed considerable activity in the presence surfactant SDS at 0.1% followed by 0.2% followed 0.5% and followed by 1% (Fig. 3-4).

*Actinomyces* cellulase showed remarkable activity and stability in the present of various commercial laundry detergents such as *Ariel*<sup>TM</sup>, *Surf Excel*<sup>TM</sup>, *Tide*<sup>TM</sup>, *Ghari*<sup>TM</sup>, *Nirma*<sup>TM</sup> and *Wheel*<sup>TM</sup> ranged from 60-40 % at 7mg/mL concentration. Cellulase showed maximum activity and stability in presence *Ariel*<sup>TM</sup> (60%) followed by *Wheel*<sup>TM</sup> (55%) followed by *Ghari*<sup>TM</sup> (50%) followed by *Surf Excel*<sup>TM</sup> and *Tide*<sup>TM</sup> (45%) and followed by *Nirma*<sup>TM</sup> (40%) (Fig. 5). Similar types of studies have been reported worldwide by various research groups [23-25].

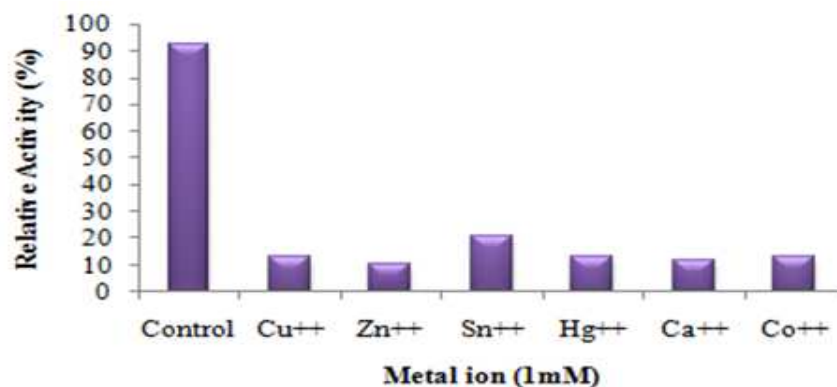


Fig. 3. Effect of heavy metal ion (at 1mM concentration) on activity and stability of cellulase

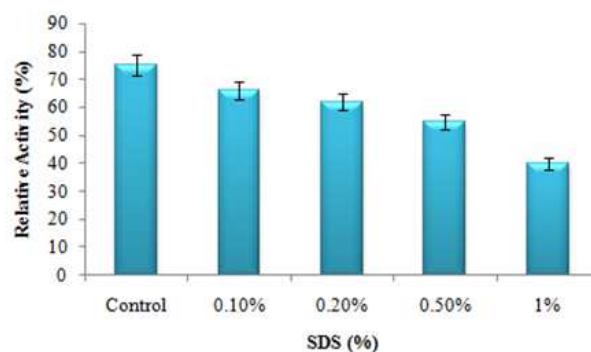


Fig. 4. Effect of SDS on activity and stability of cellulase

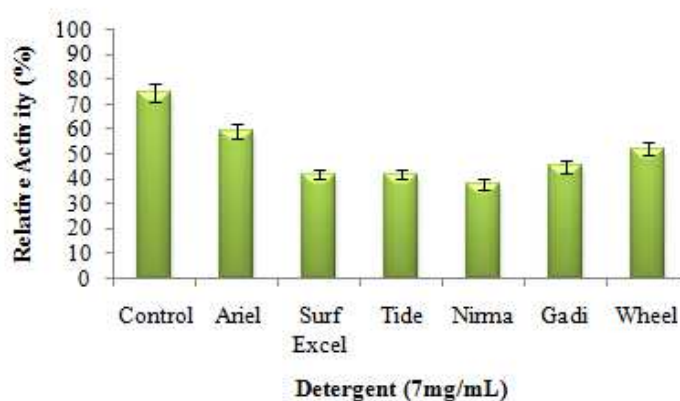


Fig. 5. Effect of commercial laundry detergent on activity and stability of cellulase

## CONCLUSION

From the result it is clear that the isolated species *Actinomyces bovis* is a potential industrially important cellulase producer. Cellulase produced by *A. bovis* was stable in wide range of temperature, pH, in the presence of different metal ions and SDS. It was showed remarkable activity and stability in the presence of various commercial laundry detergents. Hence, it is useful to improve washing performance of detergents, in bioconversion of waste biomass in agriculture and feed, food, textiles and paper industries as a catalyst.

## REFERENCES

- [1] CN Khobragade; K Sureshkumar, PS Borkar; AD Sagar, *Indian J. Chem. Technol.*, **2004**, 11, 816-819.
- [2] B, Vanholme; J de Meutter; T Tytgat; M van Montagu; A Coomans; G Gheysen, *Gene.*, **2004**, 332, 13-27.
- [3] T Kudo, *Biosci. Biotechnol. Biochem.*, **2009**, 73, 2561-2567.
- [4] M Dashtban; H Schraft; W Qin, *Int. J. Biol. Sci.*, **2009**, 5, 578-595.
- [5] S Cai; J Li; FZ Hu; K, Zhang; Y Luo; B Janto; R Boissy; G Ehrlich; X Dong, *Appl. Environ. Microbiol.*, **2010**, 76, 3818-3824. doi: 10.1128/AEM.03124-09.
- [6] F Canganella; J Wiegel, *Naturwissenschaften.*, **2011**, 98, 253-279.
- [7] MT Madigan; JM Martinko, *Brock Biology of Microorganisms*, 11th ed. USA: Pearson Educational International, Prentice Hall, **2006**.
- [8] G Lopez-Casado; BR Urbanowicz; CMB, Damasceno JKC, Rose; *Curr. Opin. Plant Biol.*, **2008**, 11: 329-337.
- [9] YH Percival Zhang; J Hong; X Ye, *Methods Mol. Biol.*, **2009**, 581: 213-231.
- [10] M Dashtban; M Maki; KT Leung; C Mao; W Qin, *Crit. Rev. Biotechnol.*, **2010**, 30, 302-309.
- [11] APHA (American Public Health Association), *Standard methods for the examination of water and waste water*. 16th Ed, Washington DC, **1985**
- [12] HR Johnsen; K Krause, *Int. J. Mol. Sci.*, **2014**, 15, 830-838.
- [13] MA Gerencser; JM Slack, *J. Bacteriol.*, **1967**, 74, 109-115.
- [14] BR Reddy; G Narasimha; GVAK Babu, *J. Sci. Ind. Res.*, **1998**, 57, 617-620
- [15] A Vyas; D Vyas; KM Vyas, *J. Sci. Ind. Res.*, **2005**, 64:281-286.
- [16] K Lin; X Kan; H Yan; D Wang, *Electro. J. Biotechnol.*, **2012**, doi:10.2225/vol15-issue3-fulltext-1
- [17] AP Pathak; BN Rekadwad, *J. Sci. Ind. Res.*, **2013**, 72, 685-689.
- [18] BN Rekadwad, *Int J. Life Sci. Biotechnol. Pharma Res.*, **2015**, 4, 26-30.
- [19] DH Bergeys; NR Krieg; JG Holt, *Bergey's Manual of Systematic Bacteriology*, Williams and Wilkins, Baltimore, **1994**.
- [20] TK Ghose, *Pure Appl Chem.* **1987**, 59, 257-268.
- [21] R Goldbeck; MM Ramos, GAG Perira; F Maugeri-Fihlho, *Bioresour. Technol.*, **2013**, 128, 797-803.
- [22] T Pirzadah; S Garg; J Singh; A Vyas; M Kumar; N Gaur; M Bala; R Rehman; A Varma; V Kumar; M Kumar, *SpringerPlus.*, **2014**, 3, 622.
- [23] R Gaur; S Tiwari, *BMC Biotechnol.*, **2015**, 15, 19. doi:10.1186/s12896-015-0129-9
- [24] SA Ladeira E Cruz; AB Delatorre; JB Barbosa; ML Leal Martins, *Electro. J. Biotechnol.*, **2015**, 18, 110-115.
- [25] SP George, A Ahmad; MB Rao, *Bioresour. Technol.*, **2001**, 77, 171-175.