RESEARCH ARTICLE

Isolation and screening of cellulolytic bacteria from soil and optimization of cellulase production

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ABSTRACT

The present investigation was undertaken to isolate and Screen the Cellulase Producing bacteria. Microbial cultures were isolated from the soil sample collected from different villages of saline belt of Akola and Buldhana District, Maharashtra India. A Total of 146 isolates were isolated and identified based on Morphology and Biochemical characterization. Among all isolated organisms 37 bacterial species were isolated, the one cellulolytic bacterial isolate shows maximum enzyme activity, and identified as *Bacillus thuringiensis*. The best conditions like pH, carbon source, temperature and incubation period were also observed for cellulase producing organisms. Carboxy methyl cellulose [1.0% (w/v)] were found to be the best carbon source for the production of cellulase and Optimum temperature and pH of the medium for the cellulase were 40°C and 7, whereas it shows maximum enzyme activity after third day of incubation period.

Keyword : Cellulose, cellulase, *Bacillus thuringiensis*

INTRODUCTION

Cellulose is commonly degraded by an enzyme called cellulase. This enzyme is produced by several microorganisms, commonly by bacteria and fungi (Immanuel *et al.,* 2006). Cellulose is the most abundant polysaccharide and renewable non fossil carbon source on earth. (Coughlin 1990). Cellulolytic microorganisms produce an array of cellulases which act synergistically to degrade cellulose (Lynd *et al*., 2002).The degradation of cellulose to glucose is effected by the cooperative action of endocellulases, exocellulases and β-glucosidases (Bhat 1997). Cellulases are the complex enzyme systems that hydrolyze the β-1, 4 glycosidic bonds in the cellulose to release glucose units (Nishida *et al*., 2007). The cellulosic enzymes required for the hydrolysis of cellulose include endoglucanases (CMCase), exoglucanases (FPase) and β-glucosidases (cellobiase) (Matsui *et al.,* 2000).

Researchers have strong interests in cellulases because of their applications in various industries, including starch processing, grain alcohol fermentation, brewery and wine, extraction of fruit and vegetable juices,

textile, detergents, animal feed, pulp and paper, as well as in research development (Gao *et al*., 2008 and Zhou *et al*., 2008).

For understanding the mechanism of cellulose degradation by cellulase, it is necessary to isolate, purify and characterize this enzyme. Therefore, the present investigation was designed to isolate and Screen the Cellulase Producing organisms from Soil.

MATERIALS AND METHODS

Isolation of organisms

Bacteria were isolated from the soil sample collected from ,different villages of saline belt of Akola and Buldhana District,Maharashtra India.

Serial dilution method was used for the isolation of cellulolytic bacteria. The medium used for cellulolytic bacteria contains 1.0 % peptone, 1.0 % carboxymethylcellulose (CMC), 0.2 % K_2HPO_4, 1 % agar, 0.03 % $MgSO_4.7H_2O$, 0.25 % $(NH_4)_2SO_4$ and pH 7. The Plates were incubated for 48 hours at 30°C.

Primary Screening for Cellulolytic Activity.

The isolated microbes were grown on basal salt media supplemented with 1% carboxy methylcellulose (Hankin and Anagnostaksis, 1975). The cultures were inoculated in the centre with almost equal amounts and incubated at 30 ± 2°C until significant growth was recorded. (Gautam *et al*., 2012). The Petri plates were flooded with Congo red solution (0.1%w/v) for 15 minutes. The Congo red solution was discarded, and the plates were washed with 1M NaCl solution allowed to stand for 15– 20 minutes. The formation of clear zone was observed around the colony when the enzyme had utilized the cellulose. (Shaikh *et al*., 2013) The clear zone around the colony was measured in order to select for the highest cellulase producer. (Gautam *et al*., 2012)

Identification of cellulolytic microbes :

Identification of cellulolytic microbes was carried out by method described by Cowen and Steel (1993) and Cullimore (2000) which was based on morphological and bio-chemical characteristics.

Secondary Screening and Inoculum development

Pure cultures of selected bacterial isolates were inoculated in broth medium containing basal salt medium containing 1% Carboxy methylcellulose (CMC) as a sole carbon source and incubated at 28 ± 2°C for 4-8 days for the fungi and 37°C for 2-4 days for bacteria. After 24h of fermentation period these vegetative cells were used as inoculum source.

Submerged fermentation process:

The Identified cellulolytic species were screened for cellulase enzyme production in submerged fermentation process**,** having composition of basal salt medium containing Carboxy methylcellulose (CMC) 1 %and it is sterilized at 121°C for 15 min. Fermentation was carried out in 250 ml Erlenmeyer flasks, each containing 100 ml sterile production medium and inoculated with 5% of standard inoculums (containing 2-3.5x106 cells/ml). The flasks were incubated at 37°C for bacteria on a rotary shaker at 150 RPM for 72h. (Shaikh *et al*., 2013)

Preparation of Crude enzyme:

After termination of the fermentation period the fermented broth was centrifuged at 1600 RPM for 20 min at 4°C to remove the unwanted material. The clear supernanant thus obtained after centrifugation served as crude enzyme source from bacteria (Shaikh *et al.,* 2013)

Estimation of Cellulase Activity by DNS Method

The selected bacterial strains were inoculated in enzyme production medium containing the following composition: 20g Carboxymethyl cellulose, 5g yeast extract 0.2g $MgSO_4.7H_2O$, 5g K_2HPO_4, 10g NaCl in 1000ml at pH7 and incubated overnight at 37°C in a shaker. After incubation, the culture was centrifuged and the supernatant was used for cellulase assay.

Enzyme Assay

Filter paper activity (FPase) for total cellulase activity in the culture filtrate was determined according to the standard method (Hankin *et al.,* 1977). Aliquots of appropriately diluted cultured filtrate as enzyme source were added to Whatman's no. 1 filter paper strip (1 × 6 cm; 50mg) immersed in one milliliter of 0.05M Sodium citrate buffer of pH 5.0. After incubation at 50°C for 1 hr, the reducing sugar released was estimated by dinitrosalicylic acid (DNS) method (Miller 1959) . One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 μmole of reducing sugar from filter paper per ml per min. Endoglucanase activity(CMCase) was measured using a reaction mixture containing 1mL of 1% carboxymethyl cellulose (CMC) in 0.5M citrate acetate

buffer (pH 5.0) and aliquots of suitably diluted filtrate. The reaction mixture was incubated at 50°C for 1 h, and the reducing sugar produced was determined by DNS method. β-glucosidase activity was assayed by the method of (Pointing 1999). One unit (IU) of endoglucanase activity was defined as the amount of enzyme releasing 1 µ mole of reducing sugar per min.

Optimization of Culture Conditions for Enzyme Production

Effect of Incubation Period on Enzyme Production

Fermentation period was an important parameter for enzyme production by *Bacillus thuringiensis*. In this study, fermentation experiment was carried out up to 7 days and production rate was measured at 24 h intervals.

Effect of Temperature on Enzyme Production

In order to determine the effective temperature for cellulase production by *Bacillus thuringiensis* fermentation was carried out at 10°C intervals in the range of 20 to 80°C.

Effect of pH on Enzyme Production.

To determine optimal pH, *Bacillus thuringiensis* was cultivated in a 150mL flask containing 50 mL optimized medium with different pH ranges from 3.0 to 8.0. The pH of the medium was adjusted by using 1N HCl or 1N NaOH. The flasks were kept in stationary stage at 37°C for 5 days of cultivation.

Effect of Temperature on Enzyme Production

In order to determine the effective temperature for cellulase production by *Bacillus thuringiensis* fermentation was carried out at 10°C intervals in the range of 20 to 80°C.

Effect of Carbon Sources on Enzyme Production

Effects of various carbon compounds namely, cellulose, CMC, dextrose, lactose, sucrose, were used for studying. The broth was distributed into different flasks and 0.5 to 2.5% of each carbon sources were then added before inoculation of the strain and after culture inoculation, the flasks were incubated for 5 days at 40°C.

RESULTS AND DISCUSSION

Screening of bacteria for Cellulase Activity

Screenings of bacteria for their cellulase activity were carried out by the hydrolysis of substrate incorporating in the basal salt medium. After an incubation period, enzyme activities were detected by the appearance of zones around the bacterial colonies. *Bacillus thuringiensis* were showed the highest zone around the colony, used for further study.

Optimization of Culture Conditions for Enzyme Production

Effect of Temperature on Enzyme Production.

The effect of temperature on cellulase activity was determined by incubating the flask at a range of temperature of 20, 30, 40, 50, 60, 70°C. The results of the test made at different temperatures value showed that the optimal temperature for exoglucanase (2.09 IU/mL) and endoglucanase activity (1.86 IU/mL) produced by *B.thuringiensis* was 40°C (Figure 1),

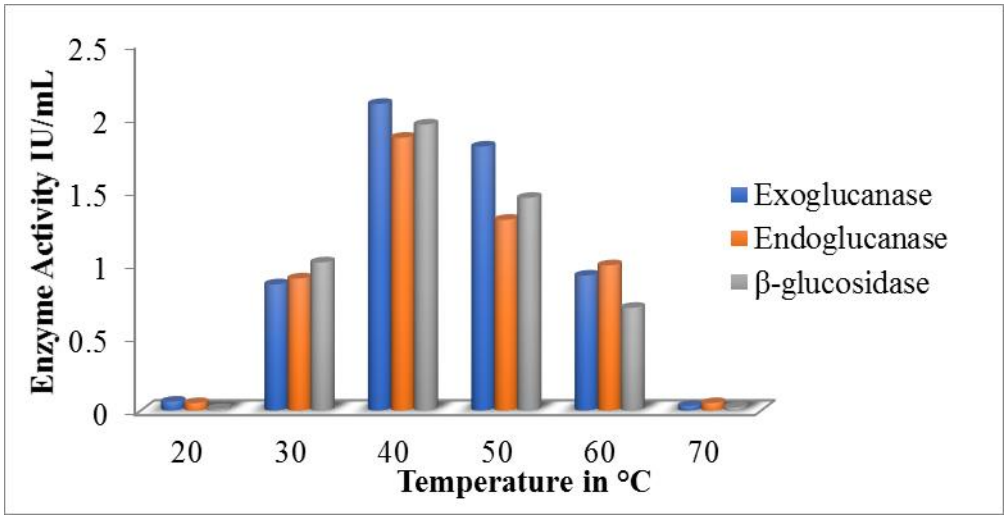

Fig.1Effect of temperature on cellulase production by *Bacillus thuringiensis* (ABS 125 A)

while the optimum temperature for β-glucosidase activity (1.95 IU/mL). Due to high temperature (above 60°C), the results showed that the enzyme activity was decreased when the temperature increased above 65°C. (Ray *et al*., (2007) reported that minimum cellulase yield was observed when fermentation was carried out at 45°C; Immanuel *et al.,* (2006) recorded maximum endoglucanase activity in *Cellulomonas*, *Bacillus,* and *Micrococcus* sp. at 40°C and neutral pH.

Effect of pH on Enzyme Production

There exists a strong influence of initial pH of the medium on enzyme production. To evaluate the effects of pH value in substrate on cellulase synthesis, the pH values were adjusted by the addition of HCl or NaOH to 3.0, 4.0, 5.0, 7.0 and 8.0. The results shown in Figures 2 showed that the production of exoglucanase (1.92I U/mL), endoglucanase (1.86IU/mL), and *β*-glucosidase (1.96 IU/mL) by *B. thuringiensis* was found at 7 pH. The enzyme activity gradually increased when increasing the pH up to the optimum followed by a gradual full in activity. Effect of pH on cellulase production by these bacteria supports the findings of Kim *et al.,* (2009). *Bacillus* subsp *subtilis* A-54 has optimum pH of 6.5 and stable in pH range of 6.5 - 8. According to previous studies, cellulases are active at the pH range of 6.0 to 7.0 from *A. niger* (Akiba *et al.,* 1995), 5.0 to 7.0 from Lysobacter sp. (Ogura *et al.,* 2006)., and 5.0 to 6.5 from Bacillus strains (Mawadza *et al.,* 2000).

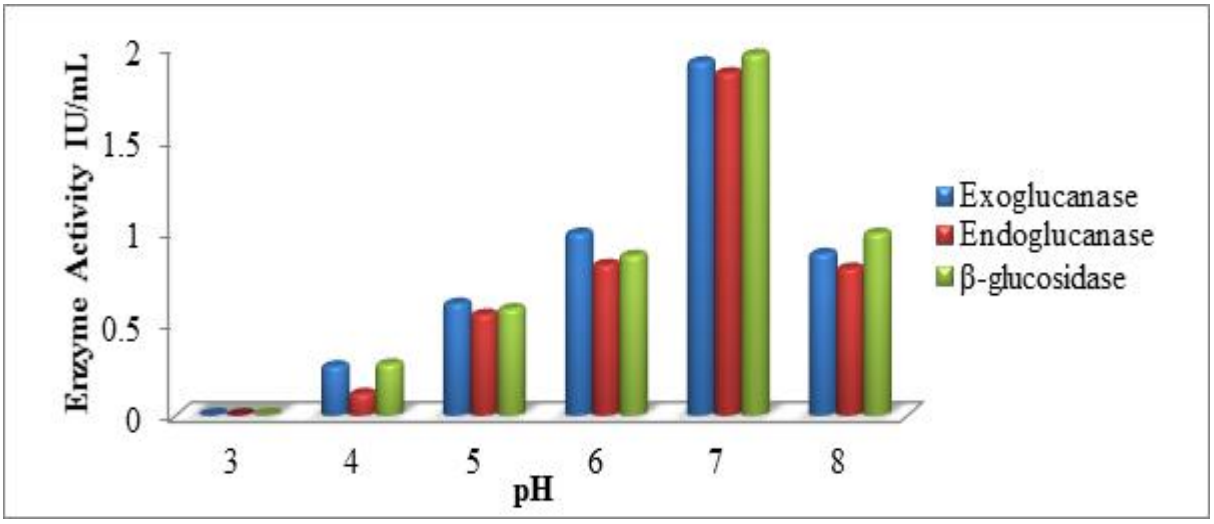

Fig.2 Effect of pH on cellulase production by Bacillus thuringiensis (ABS 125A)

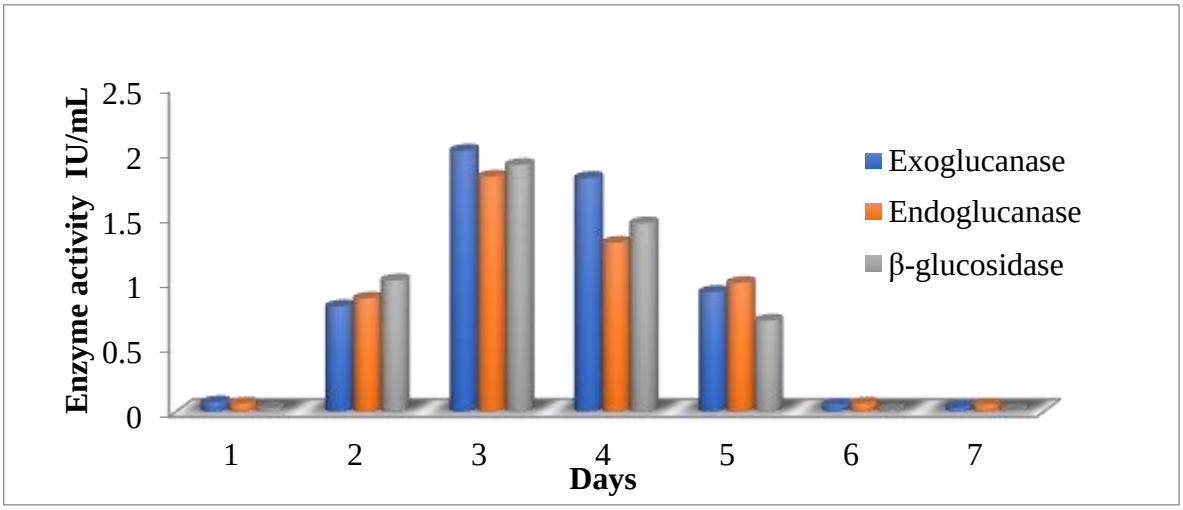

Fig 4.3(c) Effect of incubation period on cellulase production by *Bacillus thuringiensis* (ABS 125 A)

Table 1: Effect of carbon source on cellulase production by *Bacillus thuringiensis* (ABS 125 A)

Conc. %		0.5	1.0	1.5	2	2.5
Cellulose	1	0.61	1.91	1.50	0.89	0.55
	2	0.49	1.90	1.41	0.86	0.45
	3	0.41	1.85	1.39	0.81	0.49
Carboxy Methyl Cellulose	1	0.71	2.11	1.81	0.99	0.70
	2	0.69	2.02	1.71	0.97	0.62
	3	0.67	2.08	1.76	0.81	0.66
Dextrose	1	0.72	1.97	1.61	1.25	0.86
	2	0.68	1.89	1.56	1.11	0.71
	3	0.63	1.90	1.45	1.18	0.69
Lactose	1	0.41	1.83	1.51	1.11	0.71
	2	0.35	1.71	1.45	1.02	0.69
	3	0.34	1.63	1.46	1.09	0.65
Sucrose	1	0.55	1.51	1.21	0.88	0.51
	2	0.41	1.40	1.22	0.86	0.43
	3	0.40	1.49	1.22	0.76	0.42

1) Exoglucanase, 2) Endoglucanase, 3) β-glucosidase

Effect of Incubation Period on Enzyme Production.

B. thuringiensis was inoculated into basal salt medium in 150mL conical flask and incubated at 40°C for a period of 7 days. The cellulase activity was measured at regular intervals. However, the maximum yield of exoglucanase (1.41 IU/mL) and endoglucanase (1.40 IU/mL) activity was obtained after 4 days. However, maximum β-glucosidase (1.45 IU/mL) activity was shown after 4 days incubation (Figures 3). The incubation period is directly related to the production of enzyme and other metabolic up to a certain extent. The incubation periods to achieve peak cellulase activity by the *B. thuringiensis* were after 4th days, which was suitable for commercial point of view (Kang *et al*., 2004). It might be due to the depletion of nutrients in the medium which stressed the fungal physiology resulting in the inactivation of secretary machinery of the enzymes (Nochur *et al*.,1993).

Effect of Carbon Sources on Enzyme Production

Carbon sources play a vital role in the cell metabolism and synthesis of cellulase. Carbon sources tested for production of cellulase enzyme by *B. thurigiensis* were cellulose, carboxymethyl cellulose, Dextrose, Lactose, and sucrose ranging from 0.5 to 2.5% (w/v). CMC were found to be the best carbon sources for enzyme production by *B. thuringiensis* as shown in Table 1. However, the maximum production of exoglucanase (2.11 IU/mL), endoglucanase (2.02 IU/mL), and β-glucosidase (2.08 IU/mL) was obtained in culture containing 1.0% carboxy methyl cellulose. Among the different carbon sources used, the CMC was the second best carbon source (1.0%) for cellulase production by *B. thuringiensis* followed by dextrose, cellulose, lactose and sucrose (Table 1). Cellulase production increased with increases in initial sugar concentration from 1.0 to 1.5% while further increases in sugar concentration slightly reduced the yield. Utilization of CMC as carbon source is best for cellulase production as reported by Das *et al.,* (2010).

CONCLUSION

In this investigation, the culturing of *B.thurigiensis* proved to be an excellent source for the enzymes production. Cellulase producing bacteria may be found in this belt which will be helpful in production of cellulase which is industrially very important and it is further used for biodegradation of Municipal Solid Waste. Saline belt of Vidarbha region is a high potential source of cellulase producing bacteria useful in various fields such as Pharmaceutical industries and Agricultural industries.

Conflicts of interest: The authors stated that no conflicts of interest.

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