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Production of Cellulase from a Thermophilic *Bacillus* sp. Isolated from Cow Dung

Arijit Das, Sourav Bhattacharya and Lakshmi Murali

Department of Microbiology, Genohelix Biolabs, Jain University,
127/3, Bull Temple Road, Chamarajpet, Bangalore - 560019, Karnataka, India

Abstract: The objectives of the present study were isolation and identification of cellulase producing bacteria from cow dung and optimization of media and growth conditions for maximum cellulase production. Eight isolates of cellulolytic bacteria were obtained from cow dung using serial dilution and pour plate technique and pure cultures of the isolates were maintained on Carboxy Methyl Cellulose (CMC) agar slants. Screening for cellulolytic activity was performed using Congo red dye test. Biochemical characterization of the selected isolate revealed it as *Bacillus* sp. Media optimization studies showed that both Carboxy Methyl Cellulose and peptone at 2% (w/v) concentration supported maximum cellulase production. The highest enzyme yield was recorded for the media containing 20 g CMC, 20 g peptone, 1 g NaCl, 0.005 g $CaCl_2$, 0.82 g $MgSO_4$, 1.25 g K_2HPO_4, 3 g KH_2PO_4, 0.01 g $FeCl_3$, 0.005 g $ZnSO_4$, 0.0001 g $MnCl_2$ and 1 g NH_4Cl per liter of distilled water at pH 7.0 with 7% (v/v) inoculum when incubated at 42°C and 150 rpm for 4 days. The culture supernatant (crude enzyme) demonstrated a specific activity of 0.0036 $\mu molmg^{-1}min^{-1}$. This isolated strain of *Bacillus* can be used as a potential producer of thermostable cellulase which can find wide applications in various industries.

Key words: *Bacillus* sp · Cellulase · Cow dung · Media optimization

INTRODUCTION

Plant biomass is the only foreseeable sustainable source of fuels and materials available to humanity [1]. Cellulosic materials are particularly attractive in this context because of their relatively low cost and plentiful supply. It is the most abundant and renewable biopolymer on earth and the dominating waste material from agriculture [2, 3]. A promising strategy for efficient utilization of this renewable resource is the microbial hydrolysis of lignocellulosic waste and fermentation of the resultant reducing sugars for production of desired metabolites or biofuels [1]. The importance of microbial cellulose utilization in natural environments is further enhanced by the status of ruminants as a major source of dietary protein. Finally, microbial cellulose utilization is also an integral component of widely used processes such as anaerobic digestion and composting [1].

Effective bioconversion processes of cellulosic materials depend mainly on the good sources of cellulolytic enzymes, the nature of cellulosics and the optimal conditions for production and catalytic activity of the enzymes [4]. Lynd *et al.* [1] reported that for microorganisms to hydrolyze and metabolize insoluble cellulose, extracellular cellulases must be produced that are either free or cell associated. The biochemical analysis of cellulase systems from aerobic and anaerobic bacteria and fungi has been comprehensively reviewed during the past two decades. It has been recognised that thermophilic aerobic bacteria such as *Bacillus sp., Cellulomonas fimi, Pseudomonas fluorescens* and thermophilic anaerobic bacteria like *Clostridium thermocellum, Ruminococcus albus, Bacteroides cellulosolvens, Fibrobacter succinogenes* as well as some actinomycetes produce highly active cellulases [5]. Landaud *et al.* [6] screened more than 50 soil samples of various geographical origins for aerobic spore former bacteria displaying cellulolytic activity in the neutral pH range which utilize and hydrolyze amorphous cellulose when grown at pH 8 on agar solidified media. When grown in broth cultures, two different species of the *Bacillus* were shown to produce extracellular cellulolytic enzymes active in the pH 6-8 range.

Cellulases are produced commercially by several companies using submerged fermentation and find extensive applications in textile, pulp, paper, food and

Corresponding Author: Arijit Das, Department of Microbiology, Genohelix Biolabs, Jain University,
127/3, Bull Temple Road, Chamarajpet, Bangalore - 560019, Karnataka, India.
Tel: +919886919207, E-mail: jit2007das@rediffmail.com.

beverage industries. For agro-biotechnological applications a crude enzyme complex, containing cellulases, hemicellulases and pectinases are suitable. The relatively high cost of cellulases drives continuous efforts to reduce production costs. The selection of substrate depends on the intended use of the cellulase preparation [5]. The major technical limitation in fermentative production of cellulases remains the increased fermentation time with low productivity. Screening of bacteria, optimization of fermentation conditions and selection of substrates are important for the successful production of cellulase [7].

Therefore, the objectives of the present study were isolation and identification of cellulase producing bacteria from cow dung and optimization of media and growth conditions for cellulase production.

MATERIALS AND METHODS

Collection of Sample: Cow dung sample was collected using pre-sterilized petri dishes and sterile spatula from the cow shed located near the campus of Genohelix Biolabs, Bangalore, India. Precautionary measures were taken to minimize the contamination. The dung sample was mixed well and processed on the same day.

Isolation of Cellulolytic Bacteria: The cellulase producing bacterial strains were isolated using serial dilution and pour plate technique. Primary screening was performed by growing the isolates on minimal agar medium containing (g/l): K_2HPO_4, 7; KH_2PO_4, 2; $(NH_4)_2SO_4$, 1; glucose, 1; sodium citrate, 0.5; $MgSO_4$, 0.1; agar agar, 20; and distilled water at pH 7, supplemented with 1% carboxy methyl cellulose (CMC) at 37°C for 48 hours.

Identification of the Isolates: The bacterial isolates obtained after the primary screening were maintained in pure culture on CMC supplemented minimal agar slants. All the agar slants were refrigerated at 4°C until used. Study of colony morphology of the isolated cultures was carried out followed by Gram's staining. Biochemical characterization of the isolated colonies was carried out using standard protocols [8]. Identification was carried out according to Bergey's Manual (7th Ed.). All the media used during the course of the study were obtained from Himedia Laboratories Pvt. Limited (A- 406, Bhaveshwar Plaza, Mumbai-400086, India).

Secondary Screening: A secondary screening for cellulolytic activity was conducted by using Congo red test. The bacterial isolates were grown on sterile CMC agar plates containing (g/l): KH_2PO_4, 1; $MgSO_4$, 0.5; NaCl, 0.5; $Fe_2(SO_4)_3$, 0.01; $MnSO_4$, 0.01; NH_4NO_3, 0.3; CMC, 10; agar agar, 20; and distilled water at pH 7. The CMC agar plates were incubated at 37°C for 5 days to allow the secretion of cellulase. Following incubation, the agar media was flooded with an aqueous solution of Congo red (1% w/v) and left for 15 minutes. The Congo red solution was then poured off and the plates were further treated with 1 M NaCl for 15 minutes. The formation of a clear zone of hydrolysis indicated cellulose degradation [9].

Cellulase production, assay of cellulase and optimization were carried out using the selected strain in triplicates.

Cellulase Production: CMC supplemented modified mineral salts solution medium containing (g/l): CMC, 5; $CaCl_2$, 0.005; $FeCl_3$, 0.00005; $ZnSO_4$ 0.005; $MnCl_2$, 0.000126; NH_4Cl, 1; NaCl, 1; $MgSO_4$, 0.82; K_2HPO_4, 1.25; KH_2PO_4, 3; yeast extract, 5; and distilled water at pH 7, was prepared and the contents were sterilized. Each of the culture tubes containing sterile broth was separately inoculated with the isolate and incubated at 37°C for 48 hours in a shaker incubator (Orbitek) at 150 rpm. Following incubation, the cell density was recorded at 660 nm [10]. The broth was centrifuged at 14 000 x g for 30 mins at 4°C. The clear supernatant was collected separately and the cellulase enzyme assay was carried out using dinitrosalicylic acid method [11].

Cellulase Assay: Cellulase activity was assayed by using 1% CMC (S. D. Fine Chem Ltd., Mumbai, India) as the enzyme substrate. The reaction mixture contained 1 ml of the substrate solution of 0.25 g of CMC wetted with 24 ml of distilled water in a beaker and covered with aluminium foil and boiled to dissolve on hot plate at 100°C for 10 mins and made up the volume to 25 ml with distilled water. To this solution, 0.5 ml of the culture supernatant crude enzyme and 1 ml of sodium acetate buffer was added. The mixture was incubated at 37°C in water bath with shaking for 30 mins. Released reducing sugar was measured using 3, 5 - Dinitrosalicylic acid (DNSA) [11] and glucose as standard. The colour was developed by boiling in water bath for 5 mins and read using spectrophotometer at 540 nm. One unit of enzyme activity was defined as the amount of enzyme required to liberate

μg of glucose per milligram of enzyme protein per minute under the standard assay conditions. One unit of specific activity was defined as the amount of enzyme required to liberate μmol of glucose per milligram of enzyme protein per minute under the standard assay conditions.

Protein Assay: Protein was determined by the method of Lowry *et al.* [12] with crystalline bovine serum albumin as the standard.

Optimization: The optimization of media and growth conditions was carried out based on stepwise modification of the governing parameters for cellulase production. The effects of various substrates consisting of filter paper, CMC, dry leaves, hay, saw dust and coir fibre supplemented in sterile mineral salt solution at different concentrations (0.5, 1, 1.5, 2 and 2.5% w/v) were examined. Cultivation was carried out at 37°C for 6 days. The effects of various organic nitrogen sources such as yeast extract, peptone and tryptone were also examined at various concentrations (0.5, 1, 1.5, 2 and 2.5% w/v).

The influence of cultivation temperature on the cellulase production was determined at different temperatures starting from 27, 32, 37, 42 and 47°C for growth and -20 to 100°C for enzyme assay. The effects of various pH such as pH 5, 6, 7, 8 and 9 for growth and pH 5.7, 6, 6.5, 7, 7.2 and 8 for enzyme assay were evaluated.

The effect of incubation period was determined for 6 days by checking the enzyme activity after every 24 hours interval. The effect of inoculum percentage on the enzyme production was determined by varying the inoculum size as 1, 4, 7, 10 and 13% (v/v). Optimization of growth conditions for cellulase production before and after optimization considering the above parameters were also carried out. All the above readings obtained at 540 nm absorbance were represented graphically.

RESULTS AND DISCUSSION

Degradation of cellulosic materials is a complex process requiring participation by a number of microbial enzymes. Habitats that contain these substrates are the best sources to find these microorganisms. Cow dung was selected as a source for obtaining desirable cellulase producing microorganisms because there is a rich assemblage of cellulolytic microorganisms owing to the diet of the ruminants that primarily consists of huge amounts of cellulosic matter. Furthermore, its wide availability, ease of processing and cost effectiveness plays major roles in cellulase production. Eight isolates of cellulolytic bacteria were obtained from the cow dung sample collected from the cowshed located near the Institute campus. Upon Gram's staining and biochemical characterization, the isolates were found to belong to the genus *Bacillus*. Following the secondary screening (Congo red test forming reddish orange halo of the zone of cellulose hydrolysis), further work was carried out with isolate No. 7, which showed the highest production of cellulase. Pure culture of the cellulolytic strain was preserved on CMC agar slants in triplicates at 4°C. Earlier studies suggest that screening for the isolates with cellulolytic activity revealed that the spore formers were more prolific producers of the enzyme compared with the cocci-shaped isolates [13].

Prior to media optimization studies, the standard glucose was estimated by DNSA method at 540 nm.

Media optimization is an important aspect to be considered in the development of fermentation technology. Formulation of a media that is cost effective for the production of cellulase can reduce the cost of the enzyme. Cellulase is an inducible enzyme and it is affected by the nature of substrate used for production. When the effects of various carbon sources on the cellulase production were determined, carboxy methyl cellulose (CMC) was found to support maximum production of the enzyme with an enzyme activity of 3.028 $\mu gmg^{-1}min^{-1}$. A minimum activity of 0.592 $\mu gmg^{-1}min^{-1}$was noted when saw dust was used as the substrate (Fig. 1). Among the organic nitrogen sources, the highest enzyme activity of 2.910 $\mu gmg^{-1}min^{-1}$ was noted with peptone (Fig. 2). 2% concentration of CMC and peptone respectively, was found to be most effective for optimal production of the enzyme (Fig. 3). Huang and Monk [14] had reported that members of the genus *Bacillus* are producers of extracellular enzymes including amylases, proteases and carboxymethylcellulase (CMCase). Similar findings were reported by Immanuel *et al.* [15] with coir fibre as the substrate.

The cultivation temperature has a marked influence on the growth rate as well as on the level of cellulase production. In our study, the optimum temperature for microbial growth was found to be 42°C (Fig. 4), whereas the highest enzyme activity of 2.404 $\mu gmg^{-1}min^{-1}$ was recorded at 51°C (Fig. 5). In general, *Bacillus* species produce less thermostable cellulases [14].

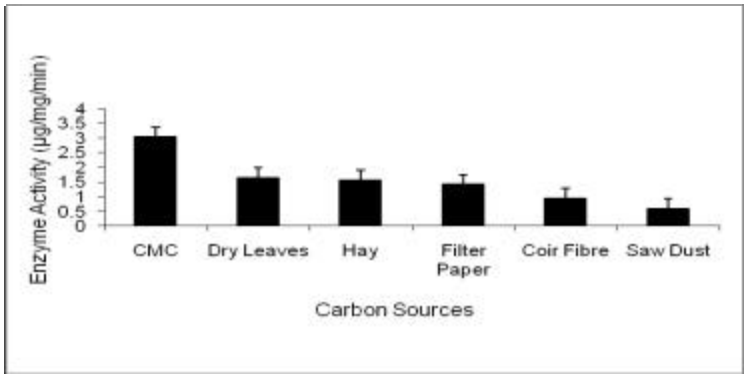

Fig. 1: Effect of different carbon sources on cellulase production

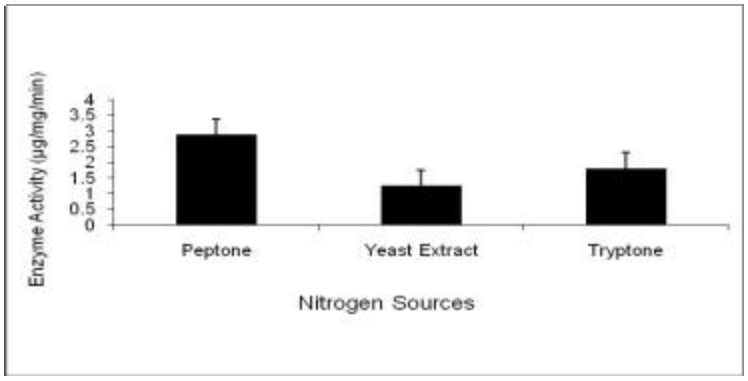

Fig. 2: Effect of different nitrogen sources on cellulase production

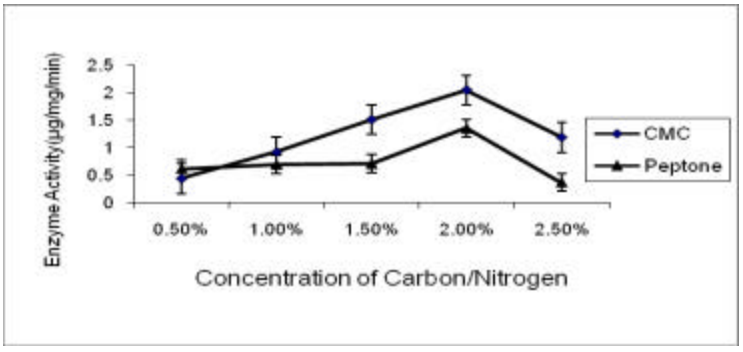

Fig. 3: Effect of different concentrations of carbon and nitrogen sources

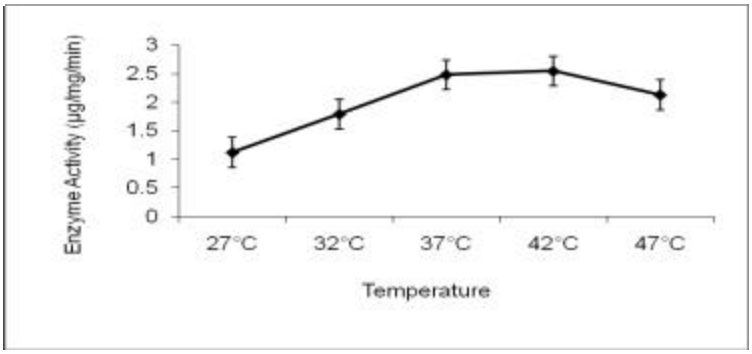

Fig. 4: Effect of temperature on bacterial growth

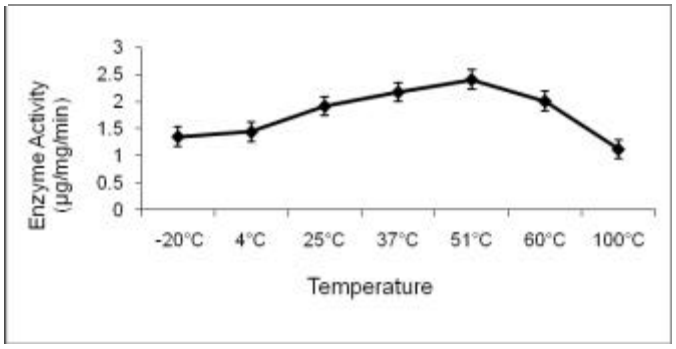

Fig. 5: Effect of temperature on cellulase activity

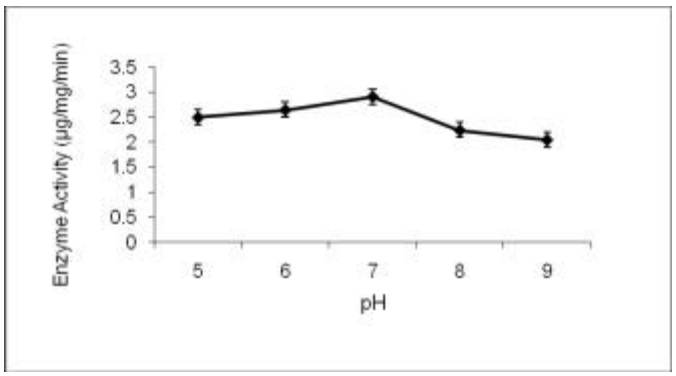

Fig. 6: Effect of pH on bacterial growth

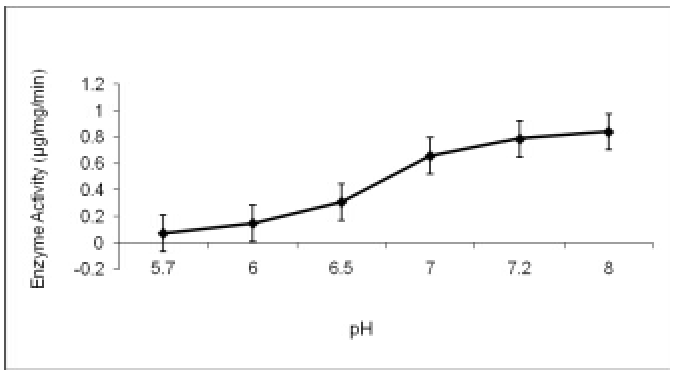

Fig. 7: Effect of pH on cellulase activity

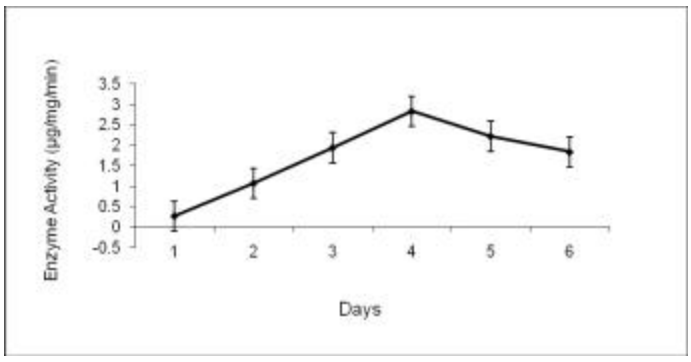

Fig. 8: Effect of incubation time on cellulase production

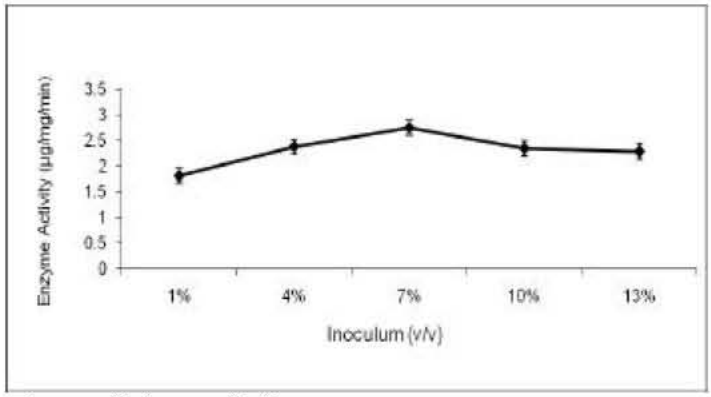

Fig. 9: Effect of inoculum size on cellulase production

Optimization of pH demonstrated optimal growth of the cellulolytic strain at pH 7 (Fig. 6), while the maximum enzyme activity of 0.838 $\mu gmg^{-1}min^{-1}$was observed at pH 8 (Fig. 7). This could probably be attributed to the alkaline environment that exists in the gut of the ruminants. From previous studies it was found that the microorganisms are capable of producing the enzyme at a broad pH range. Ariffin *et al.* [16] reported that CMCase in *Bacillus pumilus* was active at broad pH range of 5-9. Similar report was given by Mawadza *et al.* [17] in *Bacillus* species. Immanuel *et al.* [15] while using coir fibre as substrate for cellulase production, reported that the enzyme hydrolyzes substrate in the pH range of 4.0 to 9.0, with maximum activity occurring at pH 7.0. The enzyme maintained their stability over a wide range of pH 6-8, but it had maximum activity at pH 7. As compared to fungal endoglucanase, bacterial cellulase has an advantage in terms of pH optimum as buffering system is not needed in saccharification or hydrolysis of cellulose as the enzyme can optimally hydrolyze the cellulose in near neutral pH. Most of the fungal cellulase has an optimum pH of 4-6. Besides, the broad range of optimum pH (pH 5-9) is also an advantage as the enzyme can retain its activity over a wide pH range. Our result of pH optimization is in accordance with that of Immanuel *et al.* [15] and Landaud *et al.* [6].

The effect of incubation period on cellulase production was estimated for 6 days. The enzyme activity was found to increase steadily with increase in incubation time. Maximum production was observed after 4 days (2.818 $\mu gmg^{-1}min^{-1}$) while the minimum was noted at 24 h (Fig. 8). Beyond 4 days of incubation, the enzyme production substantially decreased, probably due to the depletion of essential nutrients in the media and/or accumulation of toxic secondary metabolites produced by the bacterium itself. It was interesting to observe that further incubation after 4 days reduced the growth as well as the enzyme yield.

The amount of inoculum used to culture the bacteria also affects cellulase production. The effect of inoculum size on cellulase production was examined and it was found that 7% (v/v) inoculum resulted in highest enzyme activity of 2.747 $\mu gmg^{-1}min^{-1}$ (Fig. 9). Improved distribution of dissolved oxygen and more effective uptake of nutrients contribute to the high production of the enzyme. When the inoculum sizes are too small (1%, 4%), insufficient number of bacteria lead to reduced amount of secreted cellulase. At 10 and 13% inoculum sizes, activity was found to be decreasing. Previous studies suggested that higher inoculum sizes resulted in reduced dissolved oxygen. The specific activity of the crude enzyme was found to be 0.0036 $\mu molmg^{-1}min^{-1}$.

Our results clearly indicated that the activity of cellulase produced using the thermophilic strain of *Bacillus*, was higher (0.0036 $\mu molmg^{-1}min^{-1}$) after the optimization of media and growth conditions, than that recorded before the optimization studies (0.0021 $\mu molmg^{-1}min^{-1}$). In the present study a cellulolytic strain of *Bacillus* sp. was isolated from cow dung which indicates that various cellulolytic bacterial populations representing the rumen flora can be obtained from animal dung. Using cheap natural waste materials such as dry leaves, hay, saw dust and coir fibre, the cellulolytic potential of the isolated bacterium has been explored. High growth rate of bacteria compared to fungi and ease of genetic manipulation in bacteria make them potent sources for cellulase production. This isolated strain of *Bacillus* can thus be used as a potential producer of thermostable cellulase which can find wide applications in various industries.

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