

Full Length Research Paper

# Preliminary studies on the production of endo-1,4- $\beta$ -D-glucanases activity produced by *Enterobacter cloacae*

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We report the production and characterization of endo- $\beta$ -1, 4-glucanase from isolated phytopathogenic bacterium *Enterobacter cloacae*. The bacterium was grown on different carbon sources including carboxymethyl cellulose (CMC) and 2% Avicel, for the production of endo-1, 4- $\beta$ -D-glucanases enzyme. *E. cloacae* produced maximum levels of cellulases after 96 h of fermentation. Higher levels of endoglucanases were produced when microbe was grown on CMC. Endo-1, 4- $\beta$ -D- glucanase had optimum pH and temperature of 5.8 and 40°C. The enzyme was inactivated by calcium chloride and a reducing agent  $\beta$ -mercaptoethanol.

**Key words:** Endo-1, 4- $\beta$ -D- glucanase, *Enterobacter cloacae*.

## INTRODUCTION

Cellulose, a bio-organic polymer is known to be the most abundant material on earth produced by terrestrial plants and marine algae. Cellulose is used as food, by microbes and animals, formed major part of plant structure, forming long rigid microfibrils arranged in parallel fashion joined through D-glucose units linked as  $\beta$ -linkage. The cellulose long chains are embedded in a matrix of hemicellulose and lignin (Bacic et al., 1988). The bioconversion of cellulose for the exploitation of energy is stored in the form of glucose. Many microbes and lower animals produce a battery of synergistically acting enzymes (Marsden and Gray, 1986; Klyosov, 1990; Watanabe and Tokuda, 2001; Li, 2005; Linton and Greenaway, 2004; Xue et al., 1999). A number of plants have been reported for the production of cellulases (Eshel, 2002; Walker et al., 1994). Cellulose is hydrolysed by a group of enzymes called cellulases including (i) endoglucanase or 1,4- $\beta$ -D-glucan-4-glucanohydrolases (EC 3.2.1.4), (ii) exoglucanases; including 1,4- $\beta$ -D-glucanohydrolases (cellobiohydrolases) (EC 3.2.91) and (iii)  $\beta$ -glucosidases or  $\beta$ -glucoside glucohydrolases (EC 3.2.1.21) (Marsden and Gray, 1986). Structure of a number of cellulases has been reported (Klyosov, 1990; Watanabe and Tokuda,

2001; Li, 2005; Linton and Greenaway, 2004; Xue et al., 1999).

A critical look at the structures of cellulase enzymes from different sources showed that cellulases share some common features such as catalytic domain linked to cellulose binding domain by a glycosylated proline/threonine/serine rich peptide (Gilkes et al., 1991; Klyosov, 1990) and a non-catalytic binding domain, determining the efficiency of degradation of insoluble cellulose (Klyosov, 1990). Microbial enzymes are the major source of the bioconversion of cellulose, as (1) they act as phytopathogen to generate a battery of enzymes (Andrews and Haris, 2000; Eshel, 2002; Lacy and Lukazic, 2003), (2) reside in the gut of pest insects (Schultz and Lechowicz, 1986; Schulz et al., 1986; Teera and Ferreira, 1994; Briggs and McGregor, 1996; Tokuda et al., 1997; Watanabe et al., 1997) and (3) reside in the ruminants for the digestion of cellulose (Attwood et al., 1996).

A number of reviews are available on the production, purification and characterization cloning and expression of cellulase enzymes by the microbes (Bhat, 2000; Klyosov, 1990; Marsden and Gray, 1986). Due to the biotechnological importance of these enzymes, they are considered to play a pivotal role in the degradation of cellulosic wastes. There are a few phytopathogens, studied for the production of cellulases e.g., *Erwinia*. There is a great potential for the application of microbial phyto-

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pathogen's enzymes in biotechnology, as they produce a number of toxins to percolate the tightly packed cellulose in hemicellulose, and lignin, and make it susceptible to attack. They also contribute to virulence of plant diseases. Elicitation of the disease requires the action of extracellular enzymes produced by the bacterium. *Erwinia* sp. is the only species which is considered in detail for the production of cellulases and related enzymes (Walker, 1994).

*Enterobacter* sp. has been studied by a number of workers for genomics and plant pathogenicity (Kosako et al., 1996; Lindh and Ursing, 1991; Nishijima et al., 1997). Here we report the production and characteristics of cellulases (endo-1, 4- $\beta$ -D-glucanase) produced by *E. cloacae* previously isolated from blue pumpkin beetle, *Aulacophora atripennis* (Sami et al., 2008). This microbe has not been reported for the production of cellulases before.

## MATERIAL AND METHODS

### Microorganism

The organism was isolated from blue pumpkin beetle and identified previously (Sami et al., 2008).

### Cell growth and enzymes production

CMC media was prepared in five different Erlenmeyer flasks. Each flask contained 100 ml CMC medium containing 10 g/l trypton, 5 g/l yeast extract, 5 g/l NaCl with 2 ml of 3% CMC and of 2% Avicel. The media was inoculated with 1 ml *E. cloacae* cells from 24 h grown culture.

Fermentation was carried out in 500 ml Erlenmeyer flask, each containing 100 ml of medium as given above. After inoculation, the flasks were incubated in orbital shaker at 100 rpm at 37°C. After specific hours of fermentation, samples of flasks were removed from flasks for further processes.

The supernatant was obtained by centrifugation at 10,000 rpm for 10 min. The samples were collected after 24, 48, 72, 96 and 120 h from each flask and processed.

### Protein estimation

Proteins in the samples were estimated by dye-binding method, other than described methods (Bradford, 1976).

### Enzyme assay

2 ml of 3% CMC was used as substrate mixed with 1 ml enzyme sample in 0.1 M citrate buffer solution. The flasks were incubated at 60°C for 2 h, reducing sugars thus released were estimated by the dinitrosalicylic acid reagent method (Miller, 1959). One unit of CMCCase activity was defined as amount of enzyme that released 1  $\mu$ M of reducing sugar from CMC per minute.

### Optimum pH

The optimum pH was determined by incubating the 1 ml appropriately diluted enzyme mixed with 2 ml 3% CMC in 1 ml citrate

buffer of different pH (2.6 – 8.2) for 2 h at room temperature (40°C). Reducing sugars thus released were estimated by the dinitrosalicylic acid reagent method (Miller, 1959).

### Optimum temperature

The optimum temperature of CMCCase was determined by incubating 1 ml appropriately diluted enzyme with 2 ml 3% CMC in citrate buffer (pH 5.8) at different temperature (4 – 80°C) for 2 h. Reducing sugars were estimated by the dinitrosalicylic acid reagent method.

### Effect of CaCl<sub>2</sub>

To observe the effect of CaCl<sub>2</sub> on CMCCase, different amount of 1 M CaCl<sub>2</sub> was added in reaction mixture. The reaction mixture containing 1 ml enzyme appropriately diluted, 2 ml 3% CMC and 1 ml buffer (5.8 pH) was incubated for 2 h at 40°C with addition of different amount of CaCl<sub>2</sub> (20 - 100  $\mu$ l).

### Effect of $\beta$ -mercaptoethanol

To observe the activity of CMCCase, the enzyme was incubated with  $\beta$ -Mercaptoethanol for 2 h. 1 ml appropriately diluted enzyme was mixed with 2 ml 3% CMC in citrate buffer (pH 5.8) and different concentration of  $\beta$ -Mercaptoethanol (0.1 - 1.5%) added in it. The reaction mixture was incubated for 2 h at room temperature (40°C). Reducing sugars were estimated by the dinitrosalicylic acid reagent method (Miller, 1959).

### Kinetic parameter

The activity of enzyme was observed in different concentration of substrate. The reaction mixture containing 1 ml appropriately diluted enzyme was mixed in citrate buffer with different concentration of 3% CMC (0.2-.75) and incubated at 40°C for 2 h. Reducing sugars were estimated by the dinitrosalicylic acid reagent method (Miller, 1959).

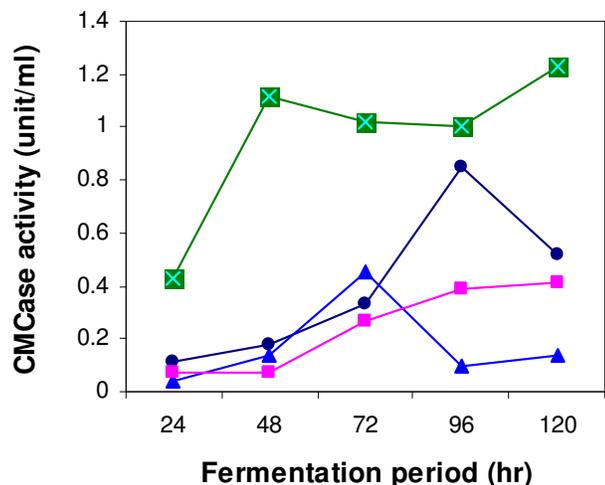
## RESULTS

### Enzymes activity

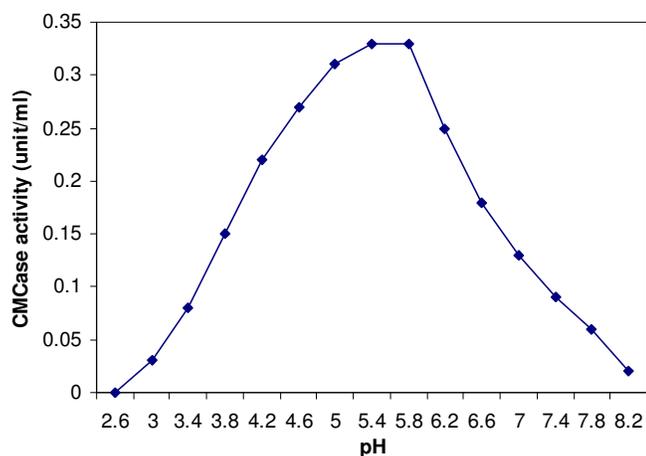
*E. cloacae* produced CMCCase in large amount when grown at 37°C on CMC as substrate. During the fermentation, the maximum activity of CMCCase was obtained after 96 h of fermentation (Figure 1) when CMC was used as carbon source while with crystalline cellulose; maximum activity was obtained after 120 h with a different profile as compared to CMC. Cellular protein was found in maximum amount in samples of 72 h of fermentation while total proteins was found maximum in samples 120 hours of fermentation (Figure 1).

### Effect of pH

The CMCCase was more active on substrate in the pH 5.8. A decline was observed at pH values below and above pH 5.8. (Figure 2).



**Figure 1.** (■) CMCase produced on Avicel, (●) CMCase produced on CMC, (▲) Cellular protein (Carbon Source CMC), and (×) Total protein (Carbon Source CMC).



**Figure 2.** Effect of pH on endo-1,4-β -D-glucanases activities produced by *E. cloacae*.

### Effect of temperature

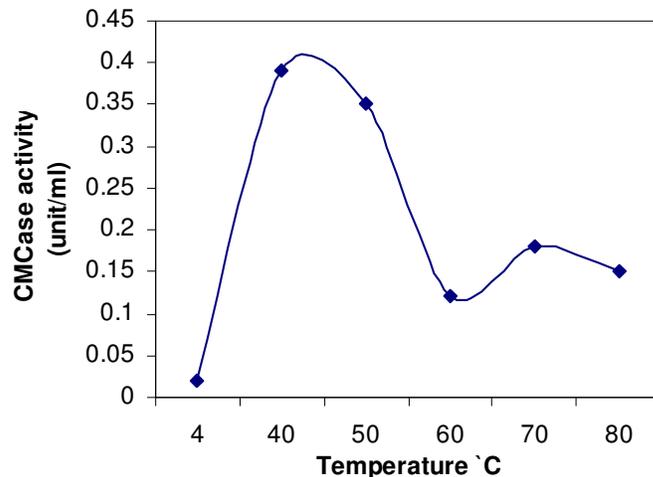
The activity of enzyme was more found at room temperature (40°C). There was a gradual decrease in the activity below and above this temperature (Figure 3).

### Effect of CaCl<sub>2</sub>

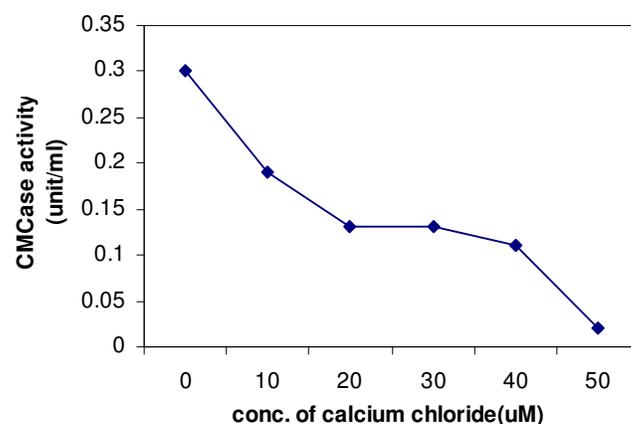
Calcium chloride was proven to inactivate the enzyme activity and at 50 μM concentration there was about 90% decrease in the enzyme activity (Figure 4).

### Effect of β-mercaptoethanol

The enzyme activity was decreased when β-mercapto-



**Figure 3.** Effect of temperature on endo-1, 4-β-D-glucanases activities produced by *E. cloacae*.



**Figure 4.** Effect of CaCl<sub>2</sub> on endo-1, 4-β-D-glucanases activities produced by *E. cloacae*.

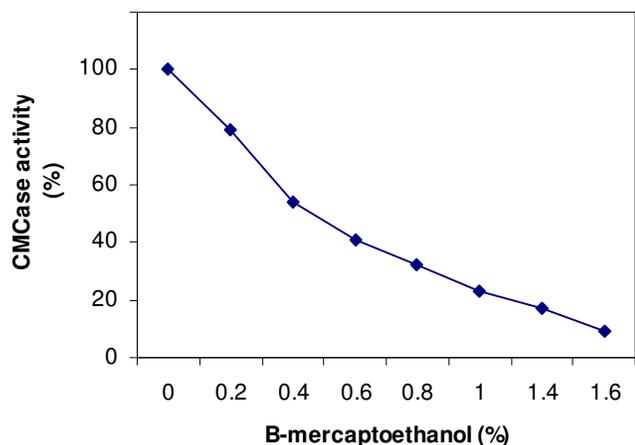
ethanol was added in the reaction mixture up to a concentration of 1.6% in the reaction mixture. Almost 90% activity was abolished when 1.5% reducing agent was added into the reaction mixture (Figure 5).

### Kinetic Parameter

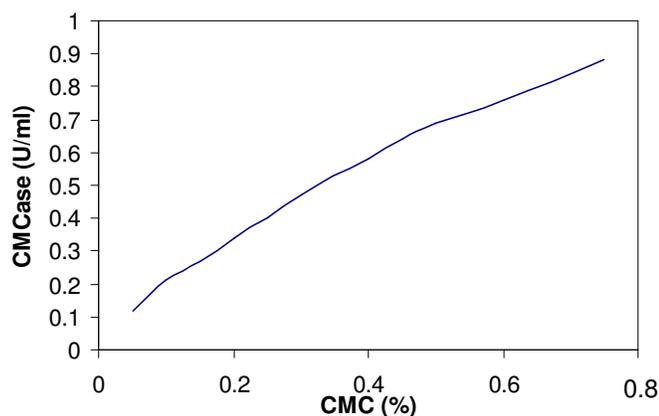
Effect of substrate concentration was studied on the enzyme activity and it was found that the enzyme showed maximum activity in the presence of 0.75% carboxymethyl cellulose as substrate (Figure 6).

### DISCUSSION

*E. cloacae* was isolated from the extract of blue pumpkin beetle *A. atripennis* as reported previously (Sami et al.



**Figure 5.** Effect of  $\beta$ -mercaptoethanol on endo-1, 4- $\beta$ -D-glucanase activity produced by *E. cloacae*.



**Figure 6.** Effect of different substrate concentration of the enzyme activity endo- $\beta$ -1,4-glucanase.

2008). The microbe was grown on CMC and crystalline cellulose for the production of endo-1, 4- $\beta$ -D-glucanase activity. *E. cloacae* was able to produce cellulase enzyme in the presence of both carbon sources. Maximum amount of enzyme produced was produced was 0.9 U/ml with CMC as compare to 0.5  $\mu$ M/ml (Figure 1) with Avicel as carbon source. There is a possibility that some of the enzyme activity remained bound to the insoluble carbon source Avicel, as it has been reported that the cellulases bound to the insoluble substrate during fermentation (Sami et al., 1988). The enzyme was active at a broad range of pH showing that it was able to hydrolyze the plant cellulose at different pathological conditions. The optimum pH of the enzyme was 5.8. Enzyme was most active at 37°C and showed a decline in activity below and above this concentration. Calcium chloride was proven to be an inhibitor, which shows the presence of a binding cleft near the active site of the enzyme. Perhaps, binding of the cleft inhibit the enzyme activity.  $\beta$ -mercaptoethanol was proven to be strict inhibitor of the enzyme indicating

the presence of di-sulphide bridges in the enzyme. The enzyme showed maximum activity when 0.75% CMC was used as carbon source. Complete characterization of the enzyme activity requires the purification of the endo- $\beta$ -1,4- glucanase.

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