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High production of cellulose degrading endo-1,4-β-D-glucanase using bagasse as a substrate from *Bacillus subtilis* KIBGE HAS☆

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ABSTRACT

Sugarcane bagasse is a cheap carbon source for endo-1,4-β-D-glucanase production as it is easily available as by-product from sugar industries. Fermentation conditions for endo-1,4-β-D-glucanase production by *Bacillus subtilis* KIBGE HAS were optimized by using un-treated sugarcane bagasse for induction of endo-1,4-β-D-glucanase and it was found that 2.0 g% bagasse in fermentation medium induced maximum endo-1,4-β-D-glucanase production. It was also found that when sugarcane bagasse was supplemented with different carbon sources, the results showed that lactose, xylose, maltose and sucrose favored endo-1,4-β-D-glucanase production, whereas cellobiose and fructose inhibit enzyme production. Maximum endo-1,4-β-D-glucanase production was obtained at 40 °C keeping the initial pH of the medium at 7.0 before sterilization. Maximum endo-1,4-β-D-glucanase production was obtained after 48 h incubation. Among different nitrogen sources, ammonium nitrate enhanced endo-1,4-β-D-glucanase production. The optimal temperature and pH for enzyme activity were 60 °C and 7.0, respectively.

1. Introduction

Cellulose is most abundant biopolymer; commonly degrade by an enzyme called as cellulase. This enzyme is produced by several microorganisms, commonly by bacteria and fungi (Bahkali, 1996; Immanuel, Dhanusha, Prema, & Palavesam, 2006; Mangelli & Forchiassin, 1999; Shin, Lee, Lee, & Park, 2000). Cellulases can be divided into three main groups of enzymes: endocellulase or endo-1,4-β-D-glucanase (E.C.3.2.1.4), exocellulase or β-exoglucanase (also called as cellobiohydrolase) (E.C.3.2.1.91), and β-glucosidase (E.C.3.2.1.21). Endo-1,4-β-D-glucanase or CMCase randomly cleaves the cellulose chain at exposed positions and create new ends, while β-exoglucanase degrades the polymeric chain from either the reducing or the non-reducing end, producing cellobiose as the main product. Endo-1,4-β-D-glucanase shows a high level of activity on soluble cellulose derivatives, such as carboxymethylcellulose (CMC) and low levels (or none at all) on microcrystalline cellulose. The first two enzymes degrade cellulose to oligosaccharides and cellobiose then β-glucosidase hydrolyses cellobiose to glucose monomers (Lynd, Weimer, Van Zyl, & Pretorius, 2002; Wood, 1989). Cellulases are used in the textile industry for cotton softening and denim finishing; in laundry detergents for color care, cleaning, and anti-deposition; in the food industry for mashing; in the pulp and paper industries for deinking, drainage improvement, and fiber modification and they are even used for pharmaceutical applications (Cherry & Fidantsef, 2003; Kirk, Borchert, & Fuglsang, 2002). Sugarcane bagasse which is a complex material is the major by-product of the sugarcane industry. Due to its abundant availability, it can serve as an ideal substrate for microbial processes for the production of value-added products (Pandey, Soccol, Nigam, & Soccol, 2000). There are many reports about the production of endo-1,4-β-D-glucanase by using sugarcane bagasse, corn stover, wheat straw and rice straw, etc. (Raimbault, 1998). Although, these raw materials are cheaper but pretreatment is generally require to improve the utilization ratio of lignocellulosic materials (Xia & Cen, 1999). These pretreatment methods are time consuming and many chemicals are required for this purpose. In addition, the overall yield of the fermentation process will be decreased because this pretreatment releases inhibitors such as weak acids, furan and phenolic compounds (Palmqvist & Hahn-Hägerdal, 2000). Therefore, in this research we used untreated sugarcane bagasse for the production of endo-1,4-β-D-glucanase and different fermentation conditions were optimized.

2. Methods and materials

2.1. Screening for endo-1,4-β-D-glucanase production

Bacillus subtilis KIBGE-HAS was grown on CMC agar plate containing (g l^{-1}): carboxy methylcellulose (CMC), 0.05; $NaNO_3$, 0.025;

☆ GenBank: EU819145.

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yeast extract, 0.01; KH_2PO_4, 0.01; NaCl, 0.01; $MgSO_4 \cdot 7H_2O$, 0.006; $CaCl_2$, 0.001; $FeCl_3$, 0.001; agar, 0.2. The pH was adjusted to 7.0 using 1.0 M NaOH.

A loop-full of growing culture of *B. subtilis* KIBGE HAS was streaked on agar plate and incubated at 37 °C for 24 h. After incubation, the plate was stained for 10 min. with 0.5% congo red and de-stained, first with 1.0 M NaCl and then further with 0.5% acetic acid, which was immediately rinsed off with cold water (Wood, 1980). The clear zones around the colonies indicate the hydrolysis of CMC by endo-1,4-β-D-glucanase.

2.2. Preparation of sugarcane bagasse powder

Sugarcane bagasse was collected from local sugar industry and washed three times with water, then dried in oven at 60 °C, until all moisture would removed. The dried bagasse was ground and its fine powder was used for further studies.

2.3. Production of endo-1,4-β-D-glucanase

Bacillus subtilis KIBGE HAS was grown in liquid medium containing ($g\,l^{-1}$): sugarcane bagasse, 20.0; lactose, 5.0; NH_4NO_3, 5.0; NaCl, 0.1; $MgSO_4 \cdot 7H_2O$, 0.6; $CaCl_2$, 0.1; KH_2PO_4, 1.0 and $FeCl_3$, 0.01. The pH of the medium was adjusted to 7.0 before autoclaving. The 10.0 ml of inoculum was transferred into 90.0 ml of sterile medium and incubated at 40 °C for 48 h. After 48 h incubation, culture broth was centrifuged (35,000 × *g* for 10 min) at 0 °C to remove the cell and supernatant was stored at −20 °C, which was used for further studies.

2.4. Enzyme assay and total protein determination

The activity of endo-1,4-β-D-glucanase (CMCase) was measured by incubating 0.25 ml succinate buffer pH 5.0, 0.25 ml enzyme with 0.5 ml 1.0% CMC (w/v) prepared in 0.05 M succinate buffer pH 5.0 and incubated at 60 °C for 20 min. Reducing sugars formed were determined by DNS method using glucose as standard (Miller, 1959).

"One unit of endo-1,4-β-D-glucanase activity was defined as the amount of enzyme which liberates 1.0 μmol of reducing sugar as glucose per min under the conditions of the assay".

Total protein was determined by the Lowry's method using bovine serum albumin as a standard (Lowry, Rosebrough, Farr, & Randall, 1951).

2.5. Time course for endo-1,4-β-D-glucanase production

Culture media flasks were incubated for different time intervals (18–96 h) at 40 °C, and after each hour of incubation endo-1,4-β-D-glucanase production was measured.

2.6. Temperature, pH and strength of buffer for endo-1,4-β-D-glucanase production and activity

Six culture media flasks were incubated at various temperatures from 25 to 50 °C for 48 h. For pH maxima, the media were adjusted from pH 4.0 to pH 11.0. All the media flasks were incubated at 40 °C for 48 h. Extracellular endo-1,4-β-D-glucanase activity was performed at different temperatures (25–70 °C).

Extracellular enzyme activity at different pH including 0.05 M glycine–HCl (3.0–4.0), acetate (4.0–5.0), succinate (6.0–6.5), potassium phosphate (6.5–8.0), Tris–HCl (7.5–9.0) and glycine–NaOH (9.0–10.0) buffer were performed at 60 °C for 20 min.

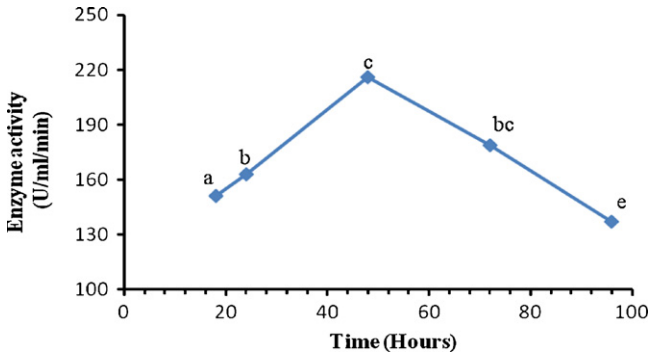

Fig. 1. Effect of fermentation time on endo-1,4-β-D-glucanase production from *Bacillus subtilis* KIBGE-HAS. Symbols (means ± SE, $n = 6$) having similar letters are not significantly different from each other (Bonferroni test, $P < 0.05$).

3. Results and discussion

3.1. Time course study for endo-1,4-β-D-glucanase production

At different time intervals, endo-1,4-β-D-glucanase production and total protein content of fermented broth are shown in Fig. 1. Highest endo-1,4-β-D-glucanase production was obtained after 48 h of fermentation. After 48 h, endo-1,4-β-D-glucanase production was decreased, which may be due to substrate consumption, another reason is the catabolite repression caused by cellobiose (Zaldívar, Nielsen, & Olsson, 2001). It might also be due to the denaturation of the enzyme, resulting from variation in pH and cellular metabolism during fermentation (Liu & Yang, 2007).

3.2. Effect of sugarcane bagasse concentration on endo-1,4-β-D-glucanase production

When untreated sugarcane bagasse was used as sole carbon source, it was found that addition of 2.0% bagasse powder in fermentation medium gave maximum endo-1,4-β-D-glucanase production with enzyme activity of 14 U/ml/min (Fig. 2). As the concentration of sugarcane bagasse was increased up to 3.0%, the total protein of the fermented medium was increased but endo-1,4-β-D-glucanase production was decreased. It has been reported that untreated bagasse is a good substrate for cellulase and biomass production as compare to pretreated bagasse. Sugarcane bagasse induces cellulase production as it contains about 50% cellulose, 25% hemicellulose and 25% lignin (Aiello, Ferrer, & Ledesma, 1996; Camassola & Dillon, 2009; Pandey et al., 2000).

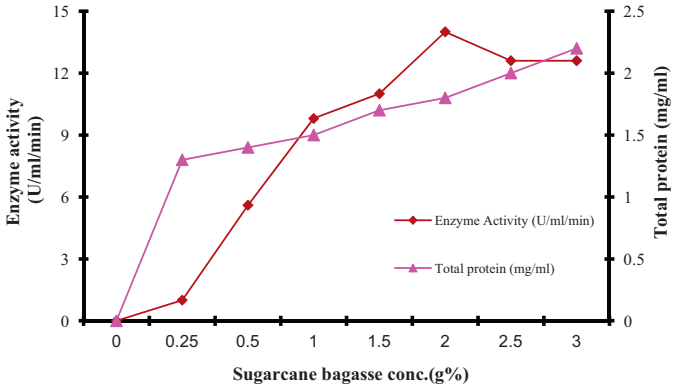

Fig. 2. Effect of sugarcane bagasse concentration on endo-1,4-β-D-glucanase production.

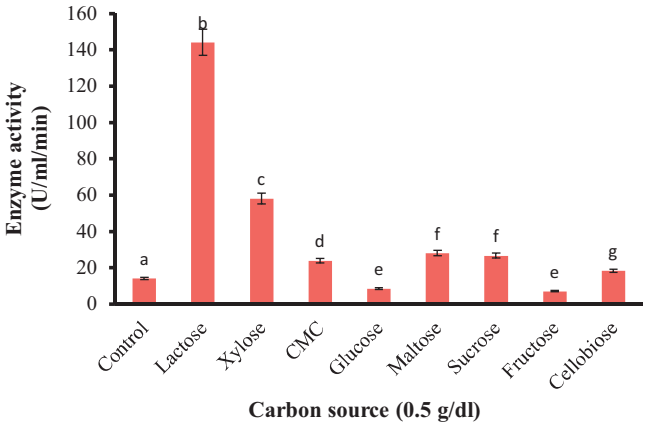

Fig. 3. Effect of supplemented carbon source on endo-1,4-β-D-glucanase production from *Bacillus subtilis* KIBGE-HAS. Symbols (means ± SE, $n = 6$) having similar letters are not significantly different from each other (Bonferroni test, $P < 0.05$).

3.3. Effect of carbon source on endo-1,4-β-D-glucanase production

It has been revealed that the cellulase is an inducible enzyme and several carbon sources have been found to efficiently promote enzyme production. Cellulose itself has been recognized as one of the best inducer, other ideal inducers include sophorose and lactose (Krishna, Rao, Babu, & Reddy, 2000; Mandels, 1975; Mandels, Parrish, & Reese, 1962; Mandels & Reese, 1957; Muthuvelayudham & Viruthagiri, 2006; Persson, Tjerneld, & Hahn-Hägerdal, 1991). In present study, when sugarcane bagasse was supplemented with different carbon sources, it was found that maximum endo-1,4-β-D-glucanase production was obtained when lactose containing medium was used with enzyme activity of 144 U/ml/min (Fig. 3). Moreover, xylose, maltose and sucrose also favored endo-1,4-β-D-glucanase production. However, addition of glucose and fructose in fermentation medium caused a reduction in enzyme production. It has been reported that high glucose suppressed the induction of cellulase, whereas low glucose stimulate enzyme production (Muthuvelayudham & Viruthagiri, 2006; Ximenes, Felix, & Ulhoa, 1996).

3.4. Effect of temperature on endo-1,4-β-D-glucanase production

Fermentation temperature is very important for bacterial growth and enzyme secretion. Therefore, cellulase production was determined from 25 °C to 50 °C. As the temperature increases, endo-1,4-β-D-glucanase production was also increased with an optimum of 40 °C (Fig. 4). After 40 °C there was a sharp decline in endo-1,4-β-D-glucanase production was observed. It may be due to fact that at low temperature, the transport of substrate across the cell is suppressed and lower yield of product is obtained. On the other hand, at high temperature, the maintenance energy requirements for cellular growth is high, due to thermal denaturation of enzymes of metabolic path way resulting in minimum amount of product formation (Aiba, Humphery, & Millis, 1973). Optimal temperature for endo-1,4-β-D-glucanase production by *B. subtilis* sub sp. *subtilis* A-53 was 30 °C (Lee, Kim, Lee, Chung, & Lee, 2010).

3.5. Effect of pH on endo-1,4-β-D-glucanase production

It is well known that initial pH of the medium effects the availability of certain metabolic ions and permeability of cell membrane. In the present study, pH of the medium was varied from 4.0 to 11.0. Production of endo-1,4-β-D-glucanase was observed in both acidic and alkaline pH and maximum production was achieved at

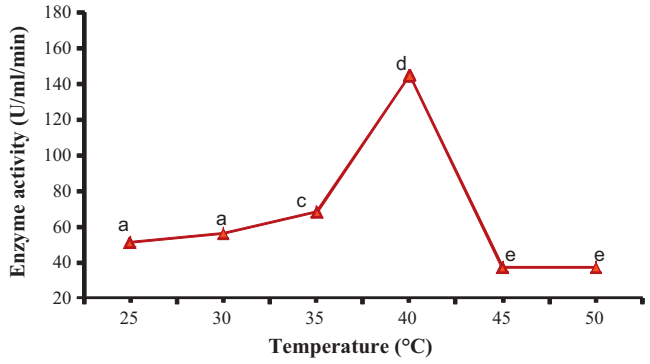

Fig. 4. Effect of fermentation temperature on endo-1,4-β-D-glucanase production from *Bacillus subtilis* KIBGE-HAS. Symbols (means ± SE, $n = 6$) having similar letters are not significantly different from each other (Bonferroni test, $P < 0.05$).

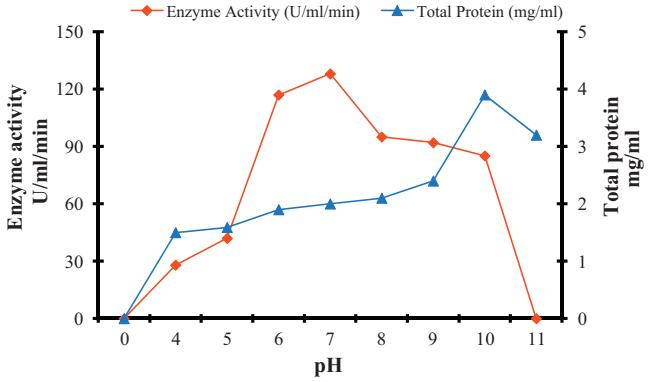

Fig. 5. Effect of initial pH of the medium on endo-1,4-β-D-glucanase production.

pH 7.0 (Fig. 5). Other investigators also reported that pH 7.0–7.5 was found more suitable for cellulase production by *B. subtilis* and *Bacillus circulans* (Ray, Bairagi, Ghosh, & Sen, 2007). Furthermore, the endo-1,4-β-D-glucanase obtained from *Cellulomonas*, *Bacillus* and *Micrococcus* sp., hydrolyzed substrate in the pH range of 4–9 with a maxima of pH 7.0 (Immanuel et al., 2006).

3.6. Effect of nitrogen source on endo-1,4-β-D-glucanase production

Different organic and inorganic nitrogen sources were used for endo-1,4-β-D-glucanase production and it was found that maximum enzyme production was achieved when ammonium nitrate was used as a nitrogen source with enzyme activity of 302 U/ml/min (Fig. 6). It has been described that ammonium compounds are the most favorable nitrogen sources for protein and cellulase synthesis (Spiridonov & Wilson, 1998). On the other hand, organic sources were found to be more suitable for cellulase production by *B. subtilis* and *B. circulans* than inorganic sources (Ray et al., 2007).

3.7. Effect of temperature on endo-1,4-β-D-glucanase activity

Extracellular endo-1,4-β-D-glucanase activity was performed at different temperatures ranging from 25 °C to 70 °C. The enzyme showed its activity in wide temperature range with optimum of 60 °C (Fig. 7). It has been described that increasing temperature has the general effect of increasing enzyme activity and optimum temperature for endo-1,4-β-D-glucanase from *B. pumilus* was 60 °C (Onsori, Zamani, Motallebi, & Zarghami, 2005; Ozaki & Ito, 1991). The enzyme retained 82% of its original activity at 70 °C. In

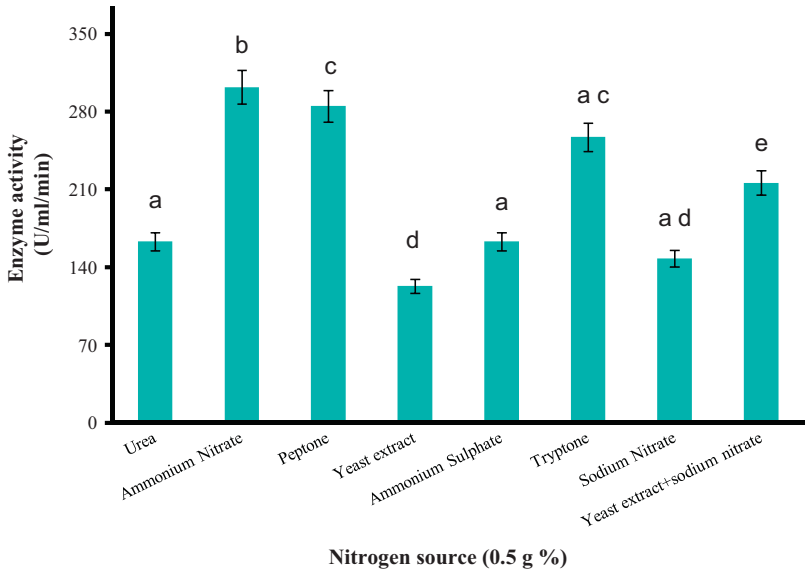

Fig. 6. Effect of nitrogen source on endo-1,4-β-D-glucanase production from *Bacillus subtilis* KIBGE-HAS. Symbols (means ± SE, $n = 6$) having similar letters are not significantly different from each other (Bonferroni test, $P < 0.05$).

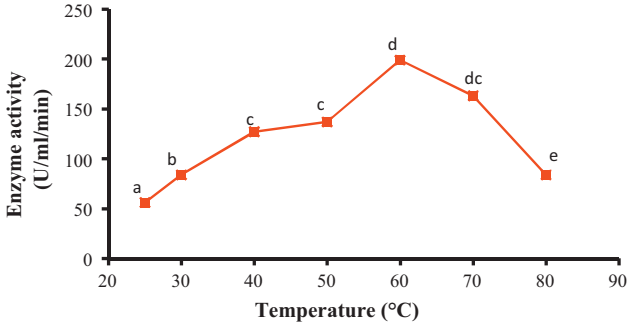

Fig. 7. Effect of temperature on endo-1,4-β-D-glucanase activity from *Bacillus subtilis* KIBGE-HAS. Symbols (means ± SE, $n = 6$) having similar letters are not significantly different from each other (Bonferroni test, $P < 0.05$).

addition, at 80 °C the enzyme activity decreased up to 42%, whereas endo-1,4-β-D-glucanase from *B. pumilus* EB3 retained only 66% of its maximum activity at 70 °C (Ariffin et al., 2008).

3.8. Effect of pH on endo-1,4-β-D-glucanase activity

Extracellular endo-1,4-β-D-glucanase showed its activity in both acidic and alkaline pH but maximum enzyme activity was detected in succinate buffer pH 5.0 (Fig. 8). It retained 73% and 66% of its maximal activity at pH 3.0 and 9.0, respectively. At pH 10.0, endo-1,4-β-D-glucanase lost its activity rapidly and only 34% activity was retained.

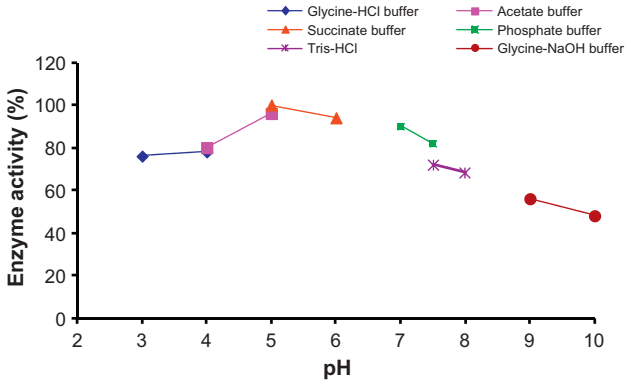

Fig. 8. Effect of different buffers having different pH on endo-1,4-β-D-glucanase activity from *Bacillus subtilis* KIBGE-HAS.

4. Conclusion

There are many renewable resources available which are being used for several industrial purposes. Among these biodegradable materials, bagasse is one of the major industrial wastes which are not only biodegradable but currently, it is also used as a primary fuel source for the production of biofuel. Use of bagasse without any chemical treatment is of major importance and cellulose present in it can be degraded using a bacterial enzyme specifically endo-1,4-β-D-glucanase.

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