ISSN 1330-9862
(FTB-2024)

preliminary communication

A Novel Thermo-Alkalitolerant Endoglucanase Production Using Cost-Effective Agricultural Residues as Substrates by a Newly Isolated *Bacillus* sp. NZ

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Received: December 24, 2007
Accepted: February 16, 2009

Summary

Highly alkalitolerant and moderately thermotolerant *Bacillus* sp. NZ, isolated from degrading lignocellulose, produced a novel highly thermotolerant and alkalitolerant endoglucanase. Maximum activity of endoglucanase was observed in the medium with the initial pH=9 (920 U/L) after 72 h of fermentation with shaking (250 rpm) at 45 °C. Growth of the organism and enzyme production displayed a precise relationship, showing maxima after 72–96 h of fermentation; however, the organism formed maximum biomass at pH=8, but produced the highest enzyme titre at pH=9. Among the examined crude substrates, wheat bran gave maximum activity of endoglucanase (1960–2280 U/L), and was followed by wheat straw (1480–1760 U/L), filter paper (1360–1450 U/L) and sawdust (1160 U/L). Soybean meal served as a suitable nitrogen source for good production of endoglucanase. The enzyme showed activity over a broad pH range (5–10), with maximum at pH=9–10 (2240–2290 U/L). Endoglucanase was highly active (2210–2300 U/L) over a broad range of temperature (50–100 °C), with maximum activity at 50 °C (2290 U/L) and at 90 °C (2300 U/L). Significantly high activity was observed even at 100 °C (91.3 %). The enzyme was highly stable at temperatures from 60 to 90 °C for a period of 30 min. Endoglucanase was purified 23-fold using ammonium sulphate precipitation. Substantial activity and stability of endoglucanase at elevated temperatures and at pH extremes indicate a potential for its successful application in industries.

Key words: endoglucanase, *Bacillus* sp. NZ, thermo-alkalitolerant, alkalitolerant, thermotolerant, wheat bran

Introduction

Cellulases have got applications in many different industries such as food, brewery, wine, pulp and paper, textile, detergent, feed and agriculture (*1*). Besides, cellulases can also be used for fermentation of lignocellulosic biomass for the production of bioethanol (biofuel), which may provide the most suitable, renewable, environmentally friendly and sustainable source of energy for the future (*2*). Three enzymes are involved in complete degradation of cellulose, *i.e.* endoglucanase (EC 3.2.1.4) cleaves the β-1,4-linkage in amorphous region of cellulose to yield long chain oligosaccharides; cellobiohydrolase (EC 3.2.1.91) acts on the reducing and non-reducing ends of oligosaccharides generated by the activity of endoglucanases to produce cellobiose, a dimer of glucose; and finally β-glucosidase (EC 3.2.1.21) hydrolyses cellobiose to yield glucose. Due to their vast applications and ever increasing demand, novel cellulases with better process suitability, high specific activity and better specificity and stability are being discovered from new lineages of cellulolytic organisms. Varieties of approaches are being used, *viz.* mutagenesis and selection, recombinant DNA technology, and exploitation of extremely high natural diversity

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to obtain organisms that can produce enzymes with desirable features. Different microorganisms including bacteria, yeast and fungi are capable of producing cellulases (*1,3–6*). Majority of studies on cellulase production have been focused on fungi, with relatively lesser emphasis on bacterial sources (*1,4–7*). Bacteria, due to their high diversity and capability to produce highly thermostable and alkalistable enzyme complement, may serve as highly potent sources of industrially important enzymes (*1,4*). In order to be employed for industrial applications, enzymes must be robust enough and highly stable under hostile conditions of industrial processes like extremes of temperature and pH. For instance, for successful application of cellulases in detergent industry, enzymes must have alkaline pH optima; similarly, for lignocellulose transformation, in pulp and paper industry or in feed industry, highly thermostable cellulases with acid/alkalistability are desirable. Many researchers have documented the production of thermostable and alkalistable cellulases from different microorganisms (*1,4,6,8*). Furthermore, it is also obvious that huge quantities of cellulases are required for their applications in diverse industries; therefore, efficient low-cost substrates must be explored for enzyme production (*9,10*). A novel endoglucanase is reported here from a newly isolated *Bacillus* sp. NZ, which showed activity at very high temperature (50–100 °C) and also possessed moderately good alkalistability (pH=8–10). Fermentation conditions were optimized for endoglucanase production using agricultural residues as the cost-effective substrates, and the enzyme was partially characterized and purified.

Materials and Methods

Chemicals and media

All the chemicals, media and medium components used in this study were of analytical grade, obtained from Sigma Chemicals Ltd., USA; HiMedia Laboratories Ltd., India; Ranbaxy Fine Chemicals Ltd., India; Qualigens Fine Chemicals Ltd., India; and Merck and Co. Inc., USA.

Cellulolytic bacteria

Cellulolytic bacteria were isolated from various natural sources like alkaline soil, degrading wood, humus, degrading lignocellulosic material, cattle manure, hay, soil under the wheat straw or rice straw, and paper industry waste. To isolate the bacteria, 5 g of the samples were inoculated into 100 mL of nutrient broth and the enrichment was carried out by incubating under shaking (200 rpm) for 24–48 h at 45 °C. The enriched broth (10 %, by volume) was inoculated into 100 mL of PY CMC medium containing (in %): carboxymethylcellulose or CMC 0.5, peptone 0.5, yeast extract 0.5, KH_2PO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.02, and NaCl 0.5, at pH=9–10 in 250-mL Erlenmeyer flasks. Incubation was done under shaking for 24–48 h at 45 °C. The enriched broth was appropriately diluted and plated on PY CMC agar (the same composition as PY CMC medium but supplemented with 2 % agar, by mass per volume). Plates were incubated at 45 °C for 48 h and developed colonies were assayed for cellulolytic activity by Congo Red staining (*11*). Colonies were flooded with 0.1 % Congo Red for 15 min and then washed three times with 1 M NaCl. Colonies showing haloes around them were picked up and purified further by streaking on PY CMC agar. A total of 21 bacterial strains were isolated and examined for their endoglucanase-producing ability in production medium (PY CMC with 1 % CMC) under submerged fermentation in shake flask at 45 °C, and the highest cellulase-producing strain (NZ) was selected for further studies.

Fermentation studies, enzyme assay and protein estimation

Submerged fermentation for enzyme production was carried out in 250 mL of production medium containing CMC (1.0 %, by mass per volume) or crude carbon sources of agricultural origin, *viz.* wheat bran, wheat straw, sawdust (teak and sheesham wood powder obtained from local timber industry) or filter paper (1 %, by mass per volume) as substrates, at varying initial pH of the medium (8–10), at 45 °C and 200–250 rpm on incubator shaker (Innova, New Brunswick, USA). The crude substrates were crushed to finer pieces (mesh size 20) and steamed for 30 min at 121 °C in the autoclave before use. The initial pH of the production medium was adjusted by using Na_2CO_3 (2 %, by mass per volume). The activated culture of bacteria was inoculated into 250 mL of production medium in Erlenmeyer flasks (500 mL) and incubated under shaking (200 rpm) at 45 °C. The enzyme activity was assayed after different time intervals. Crude enzyme was obtained by centrifugation of a suitable volume of fermentation broth at 10 000×*g* for 5 min at 4 °C (Sigma 3K30, Sigma Laboratory Centrifuge, Buckinghamshire, UK). The supernatant was considered equivalent to crude enzyme and was used for assaying endoglucanase activity after appropriate dilution.

Endoglucanase activity was assayed by using 0.5 % (by mass per volume) CMC as the substrate (prepared in Tris buffer, 50 mM, pH=8–10) at 50 °C. The released reducing sugars were measured by dinitrosalicylic (DNS) acid method (*12*) using a calibration curve for D-glucose (PerkinElmer Spectrophotometer, Model λ 35). One unit of endoglucanase activity was defined as the amount of enzyme which produces one μmol of reducing sugar (glucose equivalent) per min under given assay conditions.

Hydrolytic activity of extracellular and cell-bound crude enzyme preparation was examined on artificial substrates like 4-nitrophenylcellobioside (for cellobiohydrolase), and 4-nitrophenylglucoside (for β-glucosidase activity), and the released 4-nitrophenol was assayed spectrophotometerically (*1,7,13*).

Protein content in the supernatant was estimated by using bovine serum albumin (BSA) fraction V as standard (*14*).

Nitrogen sources for cellulase production

Yeast extract and peptone of the production medium (containing wheat bran as a carbon source) were replaced with different nitrogen sources like gelatin, urea, KNO_3, soybean meal and $(NH_4)_2SO_4$ at the rate of 0.5 % (by mass per volume), and endoglucanase titre was determined in the culture supernatant after usual fermentation in the shake flask at 45 °C.

Influence of temperature and pH on cellulase activity

The crude enzyme preparation was assayed for endoglucanase activity at different temperatures (50–100 °C). For determining the effect of pH on enzyme activity, different buffers (50 mM) were used: citrate phosphate buffer (pH=6), Tris buffer (pH=7–8) and glycine-NaOH buffer (pH=9–10).

Thermostability of endoglucanase was determined by incubating the enzyme preparation at 50–100 °C for different time periods before using it for assaying the residual activity. For determining pH stability, the enzyme was preincubated (at 45 °C) with buffers of varying pH (5–10) for different time periods and residual activity was analysed.

Partial purification of cellulase

Enzyme preparation obtained after the cultivation of the organism in production medium containing wheat bran as carbon source was subjected to ammonium sulphate precipitation of protein at different saturation levels (20–100 %). The precipitate was dissolved in a small quantity of the buffer (Tris buffer, 50 mM, pH=9) and dialyzed over night against the same buffer (buffer outside the dialysis bag was replaced with the fresh one after every 4 h). The enzyme obtained after dialysis was analysed for endoglucanase activity and total protein content.

Results and Discussion

Cellulolytic bacterium and cellulase production

Among the total of 21 bacterial strains examined, bacterial strain NZ, an isolate from degrading lignocellulosic biomass, showed the maximum endoglucanase activity on PY CMC agar, as well as under submerged fermentation. The organism was capable of growing at high alkaline pH (8–10) and at elevated temperature (45–55 °C), and was a spore former, aerobic and catalase positive. Furthermore, the bacterial strain NZ possessed the ability to hydrolyze starch, gelatin and casein. Based on the morphological and physiological characteristics, using the criteria of Bergey's Manual of Determinative Bacteriology (*15*), the organism was ascribed to the genus *Bacillus* and designated as *Bacillus* sp. NZ.

The enzyme preparation obtained after fermentation was assayed for the activity of different types of cellulases, *i.e.* endoglucanase, cellobiohydrolase and β-glucosidase, using the corresponding substrates. It was found that the enzyme preparation displayed only endoglucanase activity (as tested on CMC as the substrate) and did not show cellobiohydrolase activity (as tested on 4-nitrophenylcellobioside as substrate) or β-glucosidase activity (as tested on 4-nitrophenylglucoside as substrate). Furthermore, no detectable activity of these enzymes was present in cell-bound enzyme preparation. Thus, the organism produced only extracellular endoglucanase under the given fermentation conditions, *i.e.* in PY CMC medium at 45 °C under shaking (200–250 rpm).

Time course of endoglucanase production at various medium pH and substrates

Endoglucanase production by *Bacillus* sp. NZ was studied in the production medium containing CMC (1.0 %, by mass per volume, pH=8–10) as the substrate, in shake flask at 45 °C. Cellulase activity was low at the beginning (440–520 U/L after 24 h), but it increased later and reached maximum after 72 h of fermentation in media with different pH (Fig. 1). The highest activity of endoglucanase was observed in the medium with pH=9 (920 U/L), although a considerable activity was also seen after 48 h (720 U/L) and 96 h (760 U/L) of fermentation in the medium with the same pH. Significant

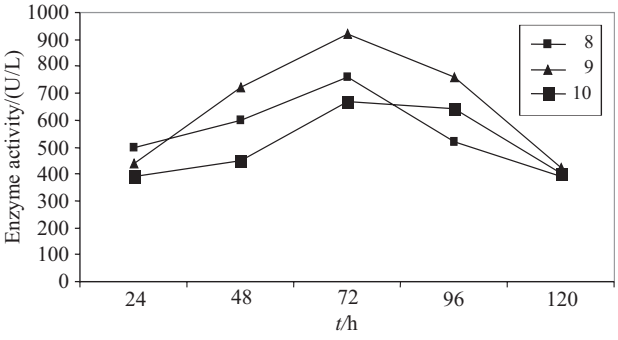

Fig. 1. Time course of endoglucanase production at various initial pH (8–10) of the medium. Fermentation was conducted under shaking at 250 rpm and 45 °C. Data represent the mean of three different experiments (three replicates)

activity was present even at pH=10 after 96 h of fermentation (640 U/L). Low endoglucanase activity at the beginning may be due to lower secretion of the enzyme during the initial stages of fermentation, and a decrease in activity after prolonged fermentation may be due to the activation of certain proteases in the medium. Maximum growth was also observed after 72 h of cultivation of *Bacillus* sp. NZ in the medium with pH=8 (Fig. 2), indicating a precise relationship between the enzyme production and biomass formation. However, maximum enzyme production was obtained at pH=9, while the maximum growth occurred at pH=8. Thus, maximum

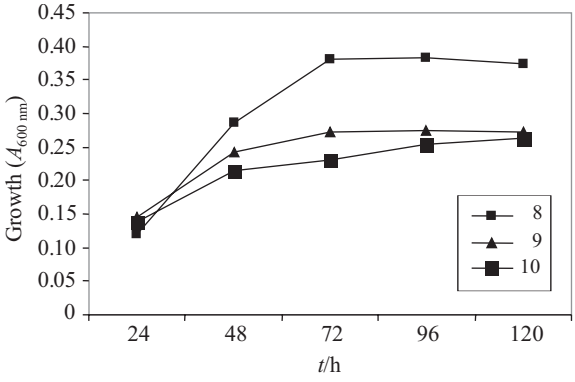

Fig. 2. Growth profile of *Bacillus* sp. NZ in the medium with various initial pH (8–10). Growth was monitored during fermentation under shaking conditions (250 rpm). Data represent the mean of three replicates

enzyme titre was reported near the end of exponential phase and early stationary phase, *i.e.* after 72–96 h of fermentation. Chan and Au (*16*) also detected CMCase activity at an early log phase of growth, which reached the maximum level at early stationary phase of growth, after 10 h of cultivation.

Nature is considered as the richest and most diverse source of industrially important microbes. There are many reports of cellulase production by bacterial species isolated from various natural sources (*6,8,13,16–20*). Optimum time for maximum enzyme production by different bacterial species reported in literature is quite variable. Different *Streptomyces* spp. have been reported to produce maximum cellulase after 72–120 h of fermentation (*8,17,20*), while *Bacillus subtilis* produced maximum cellulase after 10 h of fermentation (*16*). Maximum enzyme production stage of the organism largely depends on the type of microbial strains and their genetic make-up, and on cultural and environmental conditions employed during growth of the organism (*10*).

Pure substrates are too expensive to be employed for industrial production of cellulases, so alternative substrates, particularly crude raw materials of agricultural origin, have been explored as cost-effective substrates. CMC of the production medium was replaced with alternative substrates such as wheat straw, wheat bran, saw dust or filter paper. It was quite interesting to observe that enzyme activity was significantly enhanced on all of these crude substrates as compared to that on pure CMC. Maximum activity of endoglucanase was supported by wheat bran, *i.e.* 1960, 2000 and 2280 U/L after 48, 72 and 96 h of fermentation, respectively. Considerably good endoglucanase titre was observed on wheat straw (1480–1760 U/L) and filter paper (1360–1450 U/L) after 48–96 h of fermentation. The lowest cellulase activity was found in the medium with sawdust as carbon source (1160 U/L); nonetheless, it was higher than that reported in the medium containing CMC (Fig. 3). Optimum time period for maximum enzyme production on crude substrates was found to be 96 h of fermentation, as compared to 72 h on CMC. Generally, the easily metabolizable and utilizable substrates induce lower enzyme production (due to catabolite repression), compared to the complex and slowly utilizable substrates. Wheat bran and wheat straw possess much higher structural complexity compared to pure CMC, and therefore act as efficient substrates for enzyme induction (*10*).

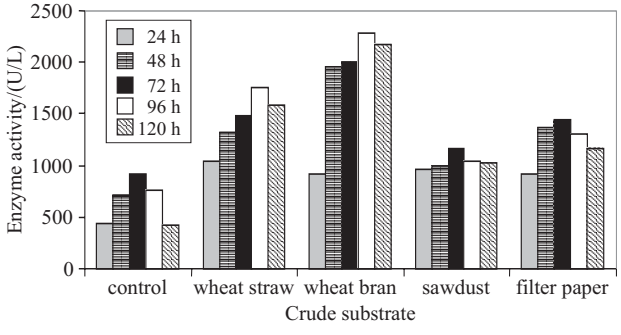

Fig. 3. Endoglucanase production using crude agricultural carbon sources. Fermentation was conducted (medium pH=9) under shaking at 250 rpm and 45 °C. Data represent the mean of three different experiments (three replicates)

Crude raw materials of agricultural origin like industrial residues from soy production, wheat bran, hay, corn cobs, *etc.* have been used for the cost-effective production of industrially important enzymes (*10,11,21,22*).

Effect of nitrogen sources on cellulase production

Nitrogen source has got profound influence on enzyme production as it is the ultimate precursor for protein biosynthesis. Besides, the nitrogen source can also affect the pH of the medium, which in turn may influence the activity and stability of the enzyme. Among the various alternative nitrogen sources tested, none was found to be suitable for endoglucanase production, except soybean meal, which supported endoglucanase activity that was comparable to that in the control (Fig. 4). Ammonium sulphate is easily metabolizable, while gelatin and urea are recalcitrant sources of nitrogen and may repress the enzyme synthesis (*11*). Yeast extract, casamino acids and proteose peptone (at 0.5 % each) have been reported to induce maximum cellulase production by *Bacillus subtilis* (*16*). Soybean meal has been reported as the best and most balanced nitrogen source for maximum cellulase and xylanase production (*11,22*).

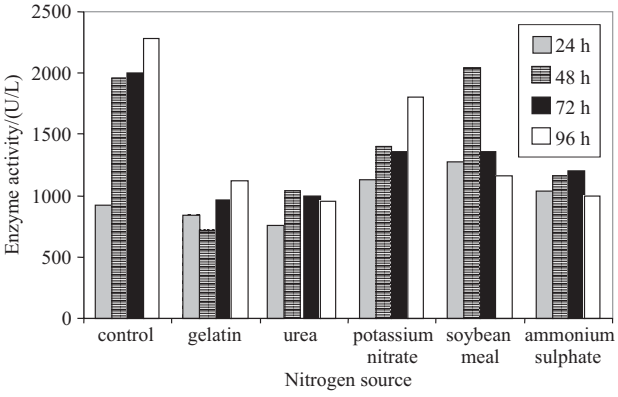

Fig. 4. Effect of different nitrogen sources on endoglucanase production. Yeast extract and peptone of the production medium were replaced with various nitrogen sources. Data represent the mean of three replicates

Partial characterization of the enzyme

The enzyme possessed activity over a broad pH range (5–10). Although maximum activity was observed at pH=9–10 (2240–2290 U/L), substantial activity was seen even under acidic pH=5–6 (1880–1990 U/L), as shown in Fig. 5. Enzyme activity decreased sharply at pH=7–8. The results indicate that the enzyme has two pH optima, or there may be different isozymes of endoglucanase which have different pH optima. Acid and alkaline stability of the enzyme were analysed by incubating the enzyme preparation with buffers of different pH (5–10) at 45 °C. Maximum stability was observed at pH=8 and pH=9. Endoglucanase was thoroughly stable for 15–30 min of incubation at pH=8–9, and retained 85–97 % of the original activity. However, at pH=10, some activity reduction was observed (residual activity 61–77 %). In the acidic range (pH=5–6), the enzyme lost considerable activity after incubation for 15 and 30 min

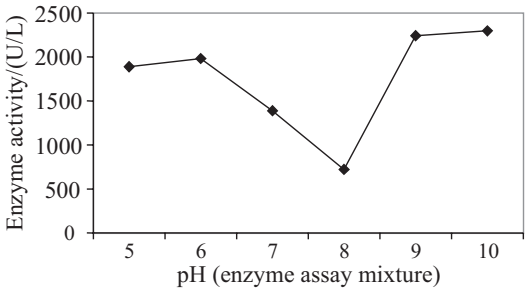

Fig. 5. Effect of pH on the activity of endoglucanase. Enzyme was assayed using different buffers: citrate buffer (pH=5–6), Tris buffer (pH=7–8) and glycine-NaOH buffer (pH=9–10)

(49–67 %). The results show that acidic pH causes more drastic activity reduction than alkaline pH after the prolonged incubation (Fig. 6). In contrast to our observations, *Bacillus licheniformis* cellulase was found to be more stable under acidic conditions (*6*). Alkalophilic *Bacillus* spp. cellulases have been reported to have optimal activities between pH=8 and pH=9, but display stability over a broad pH range (6–12) (*19,23*). However, *Streptomyces* spp. cellulases were found to be optimally active at pH= 5.5–7 (*17*). Similar to our results, a novel *Streptomyces* sp. CMCase was reported to have high activity over a broad range of pH (5–10), and remained stable for 1 h in the presence of commercial detergent (Tide, pH=11) at 40 °C (*20*). Many industrial processes are operated at pH extremes (either acidic or alkaline), therefore, the industrially important enzymes must be capable of withstanding such harsh and hostile conditions for prolonged time periods or at least during the process time. Substantial stability of endoglucanase from *Bacillus* sp. NZ at pH extremes indicates its potential industrial significance.

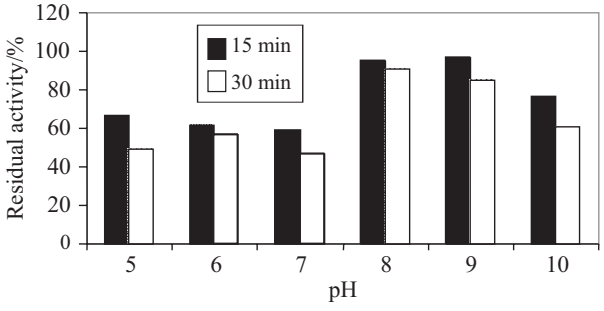

Fig. 6. Stability of endoglucanase at various pH. Enzyme preparation was incubated at 45 °C with different buffers for 15 and 30 min and residual activity was determined (original activity was 2290 U/L). Data represent the mean of three replicates

Effect of temperature on endoglucanase activity showed that the enzyme was highly active over a broad range of temperature, *i.e.* from 50 to 100 °C. There is an indication that the enzyme has got two temperature optima at which maximum activity was observed, *i.e.* at 90 °C (2300 U/L) and at 50 °C (2290 U/L), and in between the activity varied from 2210–2220 U/L (Fig. 7). Considerably high activity retention even at 100 °C (91.3 %) reflects that the enzyme is highly thermotolerant and could be of great commercial value. The most remarkable feature of *Bacillus* sp. NZ endoglucanase is that it has got activity at elevated temperatures, as most of the reported cellulases are active at temperatures much below 100 °C. Generally, microbial cellulases have temperature optima of about 50–60 °C (*19,20,23*). Thermostability of endoglucanase was examined in the temperature range of 50–100 °C. The enzyme was highly stable at temperatures from 60 to 90 °C for a period of 30 min (Fig. 8). However, incubation of enzyme above 90 °C caused reduction in the enzyme activity. Cellulases from different *Bacillus* spp. have been reported to possess optimum activity at 40–50 °C (*19,23*). However, *Bacillus licheniformis* cellulase was found to be moderately thermostable with optimum activity at 65 °C, and retained 90 % of the original activity for 1 h at 60 °C (*6*). *Streptomyces* sp. CMCase showed varying degree of stability at 50–70 °C, at pH=10 for 1–3 h (*8,20*). Highly thermotolerant enzymes are required for industrial applications, for which either the natural microflora may be screened or the enzyme may be tailored by protein engineering so that it can withstand and work at elevated temperatures during process conditions (*6*).

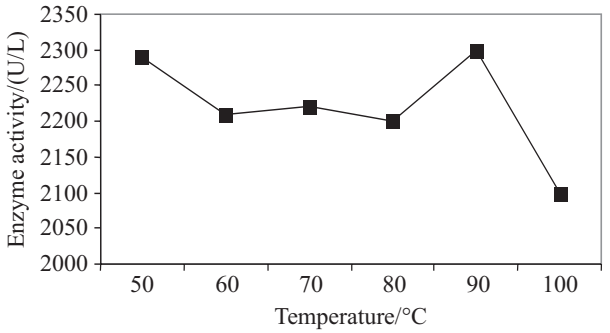

Fig. 7. Effect of temperature on the activity of endoglucanase. Activity assay was performed at various temperatures keeping the pH of the assay mixture at 9. Data represent the mean of three replicates

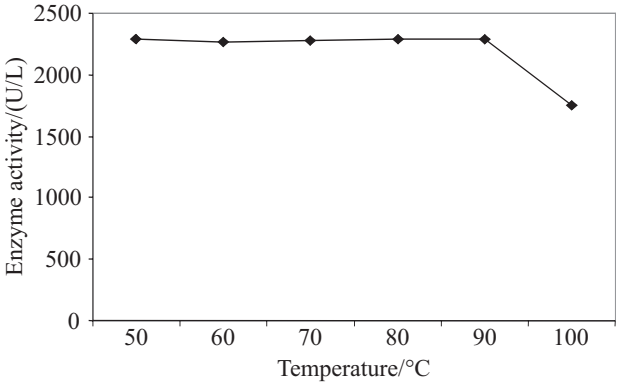

Fig. 8. Thermostability of the endoglucanase at different temperatures. The enzyme preparation was incubated for 30 min at different temperatures and then used for activity assay

Partial purification of endoglucanase

Crude enzyme preparation obtained after growing the organism in wheat bran medium was subjected to ammonium sulphate precipitation. The highest activity

was found in 40–50 % ammonium sulphate precipitated fraction. The precipitate was dissolved in a small quantity of buffer and dialyzed, and the activity and total protein assay were carried out. Ammonium sulphate precipitation resulted in the 23-fold purification of endoglucanase. Salt precipitation, gel filtration and ion exchange chromatography have commonly been used for enzyme purification from *Bacillus* spp. and other organisms (*6*,*11*,*24*).

Conclusions

It may be concluded from the results that *Bacillus* sp. NZ, a highly alkalitolerant and moderately thermotolerant organism, is capable of successfully growing at highly alkaline pH (8–10) and at moderately elevated temperature (45–55 °C). The organism produced a highly thermoactive endoglucanase (80–100 °C), which has got activity over a broad range of pH (5–10) and temperature. Furthermore, the organism successfully utilized crude carbon sources such as wheat bran and wheat straw and produced much higher titre of endoglucanase than that reported on pure substrate. This reflects the future potential of the organism for successful and economic production of thermostable and alkalistable endoglucanase. Soybean meal served as a reasonably good nitrogen source for endoglucanase production. The relatively high thermal stability displayed by the endoglucanase is a valuable characteristic for its application in processes such as enzymatic hydrolysis of cellulose and lignocellulose for the production of glucose syrup. There is a possibility that the organism has a potential to produce diverse types of cellulases with varying thermostability and alkalistability. Further study of this enzyme may provide information about the molecular basis of stability and activity of cellulases at elevated temperatures and extremes of pH.

Acknowledgements

Authors wish to thank the Head of the Department of Biotechnology, and Mrs Ekta Gupta for extending all the necessary laboratory facilities for the accomplishment of this work.

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