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OPTIMIZATION AND PARTIAL CHARACTERIZATION OF ENDOGLUCANASE PRODUCED BY *STREPTOMYCES* SP. B-PNG23

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Abstract - Streptomyces sp. B-PNG23 was selected as a promising cellulolytic strain and tested for its ability to produce cellulases from agroindustrial residues. A pH value of 7 and temperature of 28°C were found to be optimal for maximum enzyme production. The highest endoglucanase activity was obtained in a medium comprised of wheat bran (2 g/l), yeast extract (2 g/l), NaCl (2 g/l), NH_4Cl (2.5 g/l), and (0.4 g/l) of $MgSO_4$. The enzyme was active at a broad range of pH (5-8) and temperatures (40-70°C). The optimum pH and temperature were 6 and 50°C, respectively. In the presence of metal ions Mn^{2+}, Cu^{2+} and NH_4^+ the activity of the enzyme increased significantly. The enzyme retained 50% of its activity after heating at 50°C for 6 h. This enzyme could be considered as a thermotolerant biocatalyst that could be utilized in biotechnological applications.

Key words: Streptomyces, endoglucanases, optimization, characterization.

INTRODUCTION

Cellulases have attracted much interest because of the diversity of their applications. The major industrial applications of cellulases are in the textile industry for the 'biopolishing' of fabrics and for producing the stonewashed look of denims, as well as in household laundry detergents for improving fabric softness and brightness. Moreover, they are used in animal feeds for improving nutritional quality and digestibility, in the processing of fruit juices, and in baking, while de-inking of paper is yet another emerging application. A potential challenging area where cellulases could have a central role is the bioconversion of renewable cellulosic biomass into commodity chemicals (Ibrahim et al., 2007). Endoglucanases also play a key role in increasing the yield of fruit juices, beer filtration, oil extraction and in improving the nutritive quality of bakery products and animal feed. Specifications of these applications indeed suggest the necessity for a wide range of endoglucanases with varying pH and temperature optima, stability and substrate specificities. Thermostable endoglucanases active over a wide range of pH with broad substrate specificities are preferred for many of these applications.

Actinomycetes are Gram-positive filamentous soil bacteria that are the foremost producers of a large number of antibiotics and other bioactive compounds and enzymes, including important industrial enzymes involved in lignocelluloses

degradation (Jan et al., 2003). For the degradation of cellulose, hemicelluloses and lignin, which are abundant in plants, different strains of the *Streptomyces* genus have been studied, and found to be good producers of cellulase (Schrempf et al., 1995). The optimization of fermentation conditions is an important problem in the development of economically feasible bioprocesses. Thus, the expanding application of the enzyme calls for an urgent need to explore actinomycetes as a valuable source of this commercial enzyme.

In previous studies performed in our laboratory it was shown that *Jonesia denitrificans* BN-13, a new actinomycete isolated from a garden situated in Béjaia in the north part of Algeria, has a high xylanase activity. As such, this enzyme could be considered as a thermotolerant biocatalyst for use in biotechnological applications (Boucherba et al., 2010). In the present work, we describe the optimization and characterization of endoglucanase from the new strain *Streptomyces* sp. B-PNG23 as a potential cellulases producer.

MATERIALS AND METHODS

Isolation and maintenance of the actinomycete strain

During the course of screening for cellulase enzymes from actinomycetes of Béjaia habitats, an actinomycete strain was isolated and identified as *Streptomyces* sp. B-PNG23. The actinomycete was grown and maintained on Williams and Kuster medium (WK) [10g l^{-1} amidon, 0,3g l^{-1} casein, 2g l^{-1} KNO_3, 2 g l^{-1} NaCl, 2g l^{-1} K_2HPO_4, 0,05g l^{-1} $MgSO_4$ ($7H_2O$), 0,02g l^{-1} $CaCO_3$, 0,01g l^{-1} $FeSO_4$ ($7H_20$) , 1g l^{-1} glucose , 15 g l^{-1} agar, pH 7.2]. The actinomycete was cultured at 28°C for 10 days and stored at 4°C.

Nucleotide sequence accession numbers

The 16S rRNA gene sequence of the strain B-PNG23 determined in this study has been deposited in GenBank Streptomyces under accession number JQ678705.1.

Basal medium and cultivation conditions

The production of crude enzyme was carried out in carboxymethyl cellulose (CMC) containing (g/l): KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; NaCl, 1; $FeSO_4.7H_2O$, 0.01; $MnSO_4.H_2O$, 0.01; NH_4Cl, 1; (CMC), 10; yeast extract, 2. The pH was adjusted to 7.0. Each Erlenmeyer flask (500 ml) containing 100 ml of carboxymethylcellulose medium was inoculated with six discs (8 mm) cut from the periphery of actively growing colonies of 7-day-old cultures of *Streptomyces* sp. B-PNG23 on WK plates followed by incubation at 28°C for 10 days under static conditions.

Enzyme assay

Endoglucanase (CMCase, Endo-β-1,4-glucanase; E.C. 3.2.1.4) activity was determined according to Mandels et al. (1974). The reaction mixture contained 2 ml of 1% CMC in 50mM sodium phosphate buffer, pH 7.0 and 1 ml of the crude enzyme sample. The reaction mixture was incubated in a water bath at 50°C for 30 min; the reaction was terminated by adding 1.5 ml of dinitrosalicylic acid (DNS) reagent and by subsequently placing the reagent tubes in a water bath at 100°C for 5 min (Miller, 1959). One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of glucose from the appropriate substrates per min per ml of crude filtrate under assay condition.

Optimization of culture conditions for cellulase production

The cellulase production by *Streptomyces* sp. B-PNG23 was optimized following the one factor at a time approach (OFAT).

Effect of incubation period

The fermentation period was an important parameter for enzyme production by *Streptomyces* sp. PNG23. In this study, the fermentation experiment was carried out for up to 10 days and the production rate was measured at 24 h intervals.

Effect of pH

To determine the optimal pH, *Streptomyces* sp. B-PNG23 was cultivated in a 500 ml flask containing 100 ml of basal medium with different pH ranges from 4 to 10. The pH of the medium was adjusted by using 1N HCl or 1N NaOH. The flasks were kept in stationary stage at 28°C for 7 days of cultivation.

Effect of temperature

In order to determine the effective temperature for cellulase production by *Streptomyces* sp. B-PNG23, the fermentation was carried out at 25, 28, 30, 35, 40, 45 and 50°C for 7 days.

Effect of carbon sources

The effect of various carbon sources on endoglucanase production was assessed by culturing the isolate in a basalt medium (pH 7.0) at 28 °C. Walseth cellulose, cellobiose, Avicel , glucose, carboxymethylcellulose, birchwood xylan, maltose, starch, wheat bran, barley, beet pulp, orange bark, olive grignon ,wheat straw, wood sawdust and sugar cane were used as carbon source (1% w/v) individually in liquid medium. After 7 days of culture growth the endoglucanase activity was estimated.

Effect of culture medium

The concentrations of nutrients such as magnesium sulfate (0-0.6 g/l), NH_4Cl (0-5 g/l), yeast extract (0-4 g/l) and NaCl (0-5 g/l) in the culture medium were varied to optimize cellulases production.

Data presented give the average of three replicate experiments.

Partial characterization of endoglucanases

The optimal temperature was determined by assaying the enzyme activity between 30°C and 80°C, and by incubating the enzyme along with the substrate for 30 min at the respective temperatures. The optimal pH was assessed by measuring relative endoglucanase activity using 2% (w/v) CMC at different pH. Three different buffers (50mM) were used: citrate buffer for pH 4-6; phosphate buffer for pH from 6 to 8: Tris-HCl buffer for pH 8-10. The thermal stability was determined at temperatures 30, 40, 50 and 60°C after incubation in the absence of substrate for different times (from 0 to 10 h). Endoglucanases activity was examined at 5 mM concentrations of metal ions: Na^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, NH_4^+, Hg^{2+}, K^+, Ni^{2+}, Sr^{2+} and Co^{2+}, in chloride form and sulfate. Ions were added to the reaction mixture containing 2% CMC. The effects of ethylene diamine acetate (EDTA), sodium dodecyl sulfate (SDS), dithiothreitol (DTT) phenylmethylsulfonyl fluoride (PMSF) were examined in the same reaction conditions. The relative activity of 100% is expressed as the activity observed in the absence of metal ions and chemical reagents.

SDS-PAGE and zymogram analysis

SDS-PAGE was performed in a 10% (w/v) polyacrylamide gel (Laemmli, 1970). The *M*r of endoglucanases was determined using the plot of log *M*r of the standard protein markers (SIGMA Wide Range ref 58445) vs their relative mobility. The crude enzyme samples were boiled at 100 °C for 5 min and subjected to SDS-PAGE stained with a Biorad Silver Stain Plus Kit. A zymogram with 2% CMC was performed by denaturing enzyme samples with 1% SDS in Tris HCl buffer (0.05 M, pH 6.8). To avoid the possibility of incomplete denaturation, samples were treated at 100°C for 5 min. After separation of the enzyme samples by SDS-PAGE, the gel was washed at room temperature with 25% isopropanol for 5 min to remove the sodium dodecyl sulfate and renature the enzyme. Then the gel was washed four times during 30 min with 50 mM phosphate buffer pH 6 at 4°C. The washed gel is incubated at 50°C for 30 min for expression the endoglucanase activity. The gel was stained in a 0.1% (w/v) Congo red solution for 30 min before destaining with l M NaCl. The gel was immersed in acetic acid solution 0.5%, and then clear hydrolysis zones appeared against a dark blue background.

RESULTS AND DISCUSSION

In the present study, a new actinomycete strain isolated from soil samples from Béjaia (Algeria) was selected as the major cellulytic bacterial strain for cellulase enzyme production. Major impediments to exploit the commercial potential of cellulases are the yield stability and cost of cellulase production. Therefore, research should also aim at exploiting the commercial potential of existing and new cellulase in nature (Coral et al., 2002). *Streptomyces* species have always been a source of thousands of bioactive compounds. Enzymes are one of the important products of this unusual group of bacteria. *Streptomycetes* sp. with potential cellulolytic activity produced endoglucanase in liquid culture (Chellapandi et al., 2008).

Optimization of culture conditions for enzyme production

In this study, the production of cellulase by *Streptomyces* sp. B-PNG23 was optimized following the one factor at a time approach (OFAT). Deswal et al. (2011) used the same technical approach to optimize cellulase production by the fungus, *Fomitopsis* sp. RCK2010. Cellulase activity was measured at regular intervals. The maximum yield of endoglucanase (1.12 U/ml) activity was obtained after 7 days (Fig. 1). Prolonged incubation periods (7 days) were required to obtain maximum enzymatic production by streptomycetes. This is in agreement with Arunachalam et al. (2010). On the other hand, the production of the CMCase by *E. coli* JM109/DL-3 in submerged fermentations took 3 days, which resulted in an increase in productivity of CMCase and decrease in its production cost (You-Jung et al., 2012).

Temperature and pH values were found to be important parameters that influenced enzyme production (Odeniyi et al., 2009; Surinder et al 2012). Growth medium pH strongly influences many enzymatic reactions by affecting the transport of a number of chemical products and enzymes across the cell membrane (Liang et al., 2009; Kapoor et al., 2008). Our results also confirmed that growth medium pH was an important factor affecting the CMCase activity. The strain showed a greater cellulase production at pH 7. Enzymatic activity was also observed at alkaline pH (8-9) (Fig. 2a). The highest cellulase activity was observed when the strain grew at 28°C, (Fig. 2b). Jaradat et al. (2008) found that CMCase enzyme from *Streptomyces sp*. (strain J2) was active over a pH range of 4-7 with maximum activity at pH 6. However, Solingen et al. (2001) studied the alkaline novel *Streptomyces* species isolated from east African soda lakes that showed an optimal pH of 8.

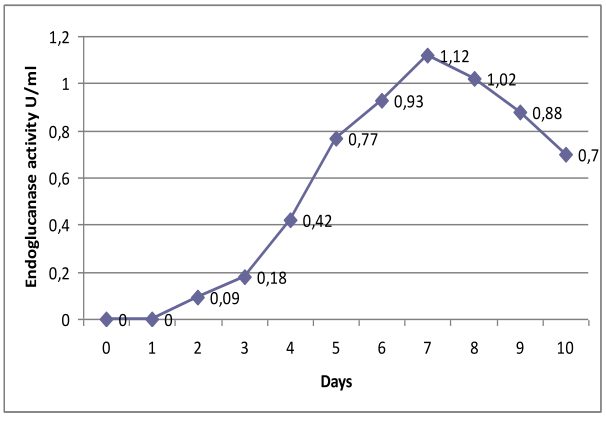


Fig. 1. Effect of incubation period in endoglucanase production

Among carbon sources, wheat bran, carboxymethylcellulose, sugar cane, glucose and maltose were the best carbon sources for enzyme production (1.20, 1.11, 0.83, 0.79, 0.76 U/ml respectively), with better production obtained by wheat bran (Fig. 3). A major carbon source for the production of fungal CMCases by *Aspergillus* and *Trichoderma* species was reported to be wheat bran (Jecu, 2000; Lee et al., 2001; Maijala et al., 2012; Gomathi et al., 2012). Although some aerobic *Bacillus* species have been shown to produce endoglucanases that can degrade amorphous cellulose, most of them cannot degrade crystalline cellulose efficiently (Bischoff et al., 2006). In a recent report, the *A. fumigatus* strain FBSPE-05 was found to produce a maximal level of endoglucanase (0.35 units/ml) in the presence of sugarcane bagasse under submerged culture (Grigorevski-Lima et al., 2009), and showed expression of six endoglucanase isoforms.

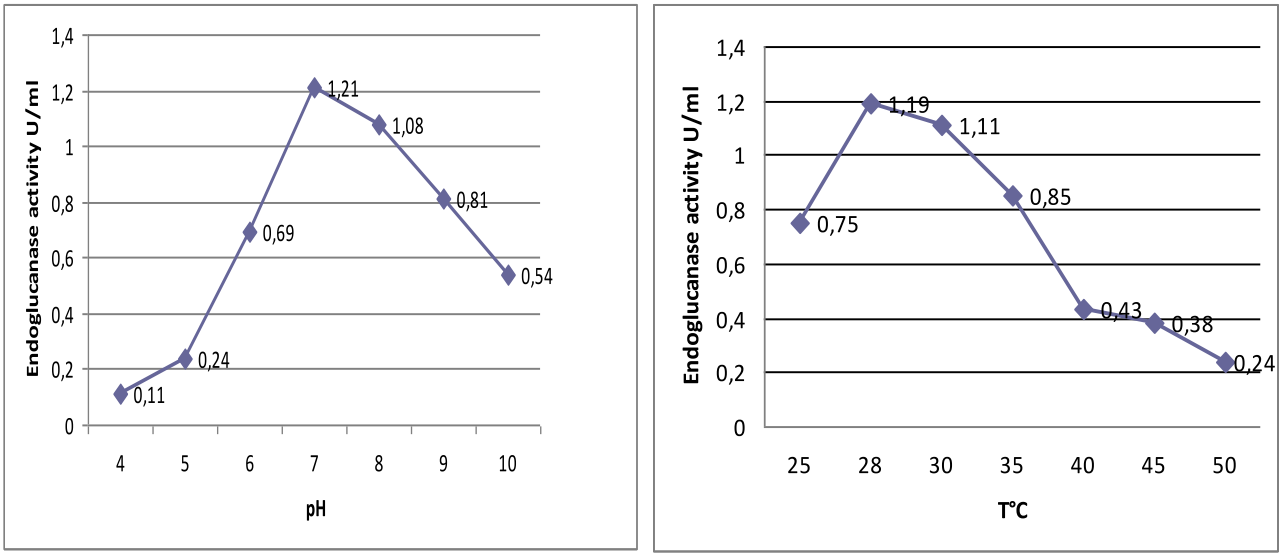


Fig. 2. Effect of initial medium pH (a), temperature (b) on endoglucanase production.

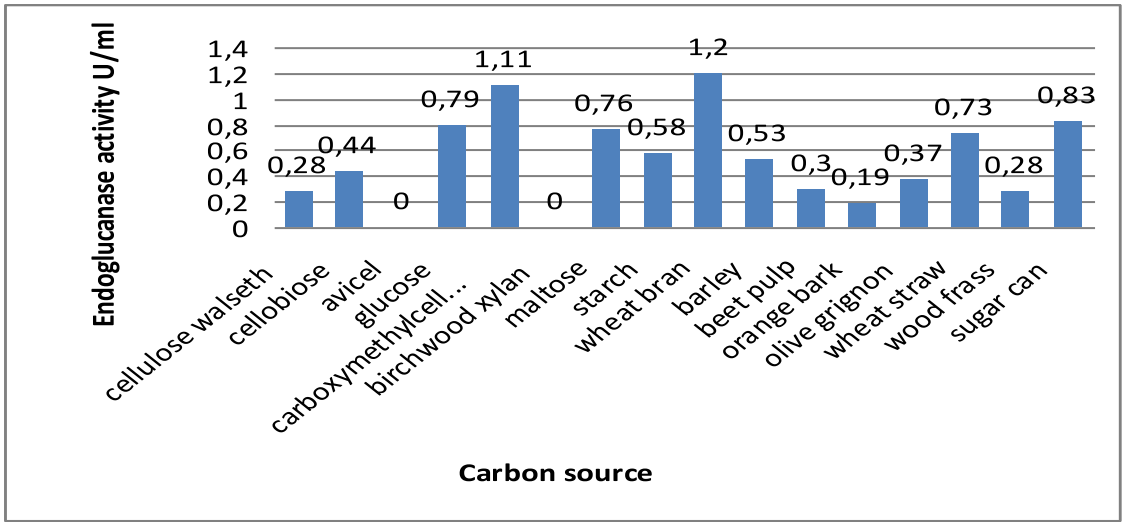


Fig. 3. Effect of carbon source on endoglucanase production

Nutrient concentrations in the growth medium influenced the production of cellulases by the bacterium. *Streptomyces* sp. B-PNG23 showed the highest cellulase production when the growth medium was supplemented with yeast extract (2 g/l), sodium chloride (2 g/l), ammonium chloride (2.5 g/l) and magnesium sulfate (0.4 mg/ml) (Fig. 4). Cellulase activity by *A. terreus* was enhanced with the addition of calcium chloride (3 mM) and magnesium sulfate (5 mM) in the medium as reported by Shahriarinour et al. (2011).

Properties of the endoglucanases

The effect of temperature on endoglucanase activity is illustrated in Fig. 5a. The optimum temperature was 50°C; at 60°C a relative activity of 95.84% was noted. When the temperature was 80°C, the relative activ-

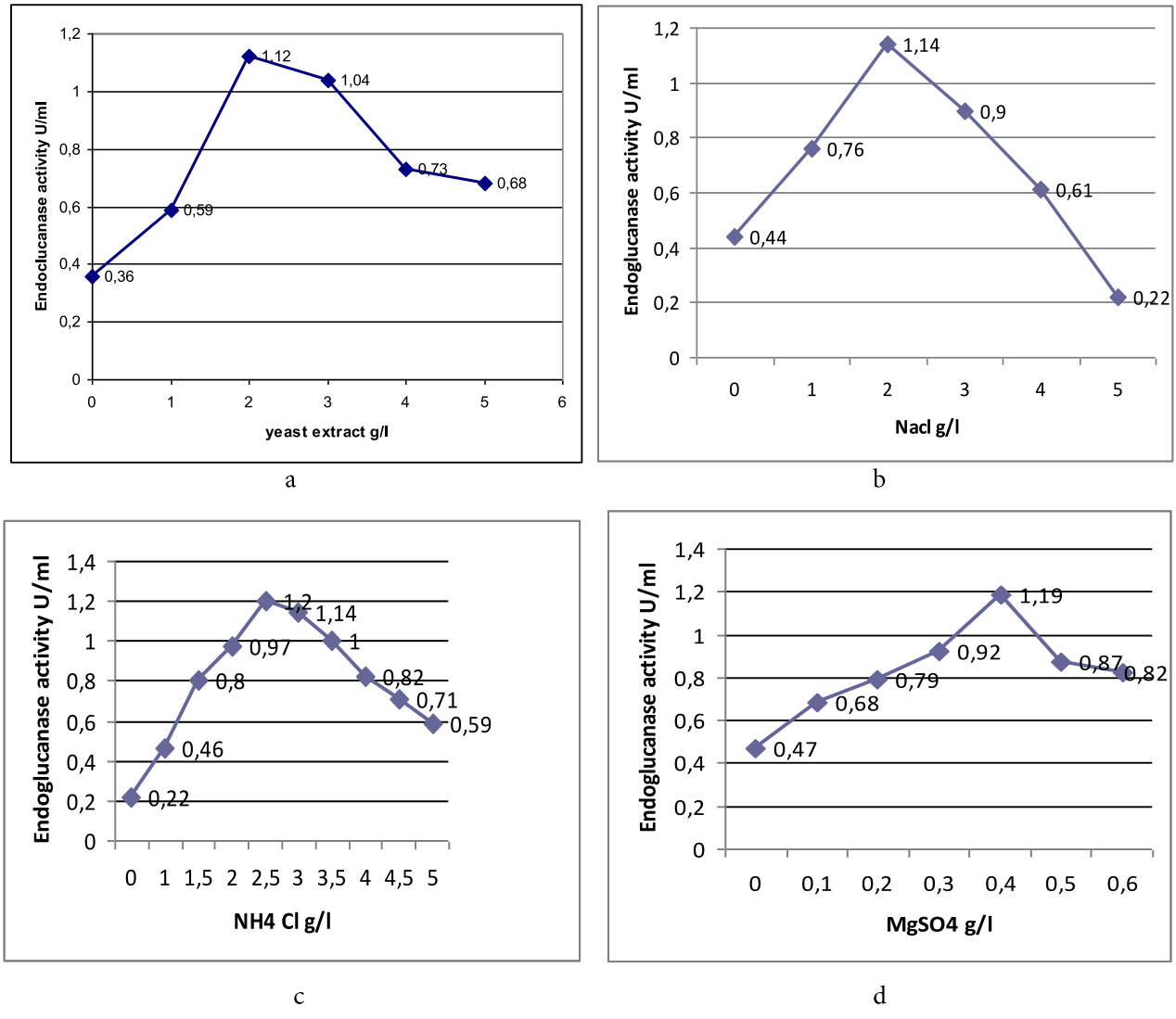
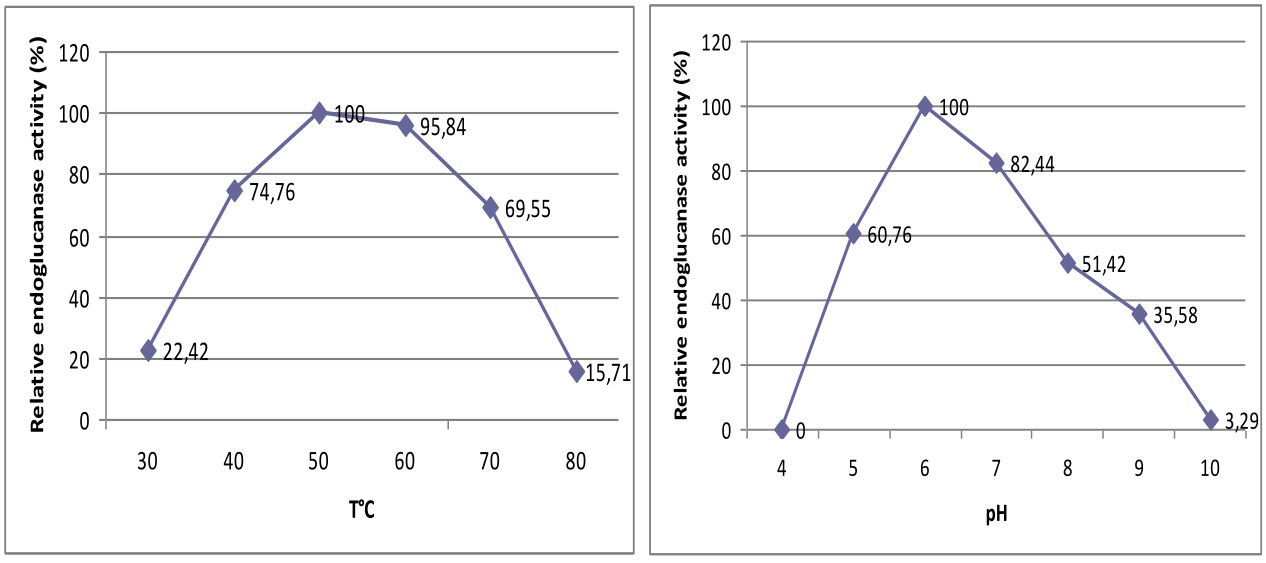


Fig. 4. Effect of yeast extract (a), sodium chloride (b), ammonium chloride (c) and magnesium sulphate (d) on the production of endoglucanase after 7 days of fermentation in shake flask culture containing 10g wheat bran at 28°C.

ity was 15.71%. Some studies have reported optimum temperatures of 60 and 65°C for the endoglucanases of *Thermococcus aurantiacus* and *Sporotrichum thermophile* (Raza et al., 2008). Jang et al. (2003) described a CMCase produced by *Streptomyces* T3-1 with an optimum temperature of 50°C, whereas the optimum temperature of native endoglucanases of *Trichoderma reesei* was found to be 55°C (Sutanu et al., 2012).

Regarding pH, endoglucanase was active between pH 5.0 and 9.0 with an optimum at pH 6 (Fig. 5b). The same range of pH optima was reported for the thermophilic endoglucanase fungus, *Chaetomium thermophile* var. *coprophile* (Ganju et al., 1990). Cellulase activity between pH 6.0 and 10.0 is useful in the textile industry (Kochavi et al., 1990) and in detergents (Suominen et al., 1993).



a b

Fig. 5. Influence of temperature (a) and pH (b) on endoglucanase activity. Data are the average of three replicates

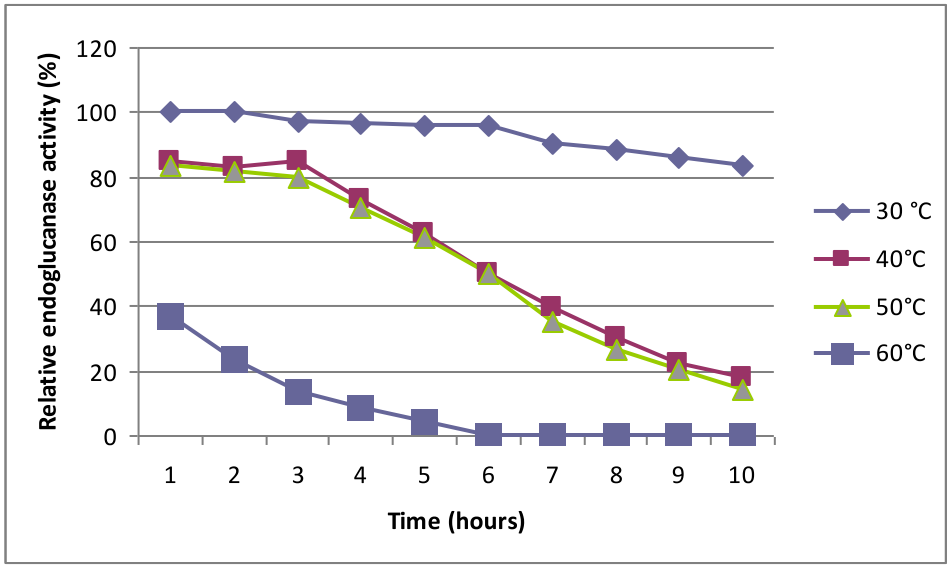


Fig. 6. Thermostability profiles of endoglucanase (without substrate): ♦: **30°C,** ▪ **: 40°C,** ▲**: 50°C,** ■**: 60°C.**

Thermal stability was assessed by preincubating endoglucanases up to 10 h at different temperature in the range of 30-60°C (Fig. 6). At 30°C, there was no significant decrease in endoglucanase activity during 10 h. The enzyme was stable at 50°C, with a half-life of 6 h; it was sensitive at 60°C and only an activity of 4.2% was observed after 5 h exposure. The results clearly indicated that the suitable temperature range for an industrial application for endoglucanase from *Streptomyces sp. B-PNG23* was 30-50°C. In an earlier study, a thermophilic *Bacillus* strain was shown to produce maximum cellulase

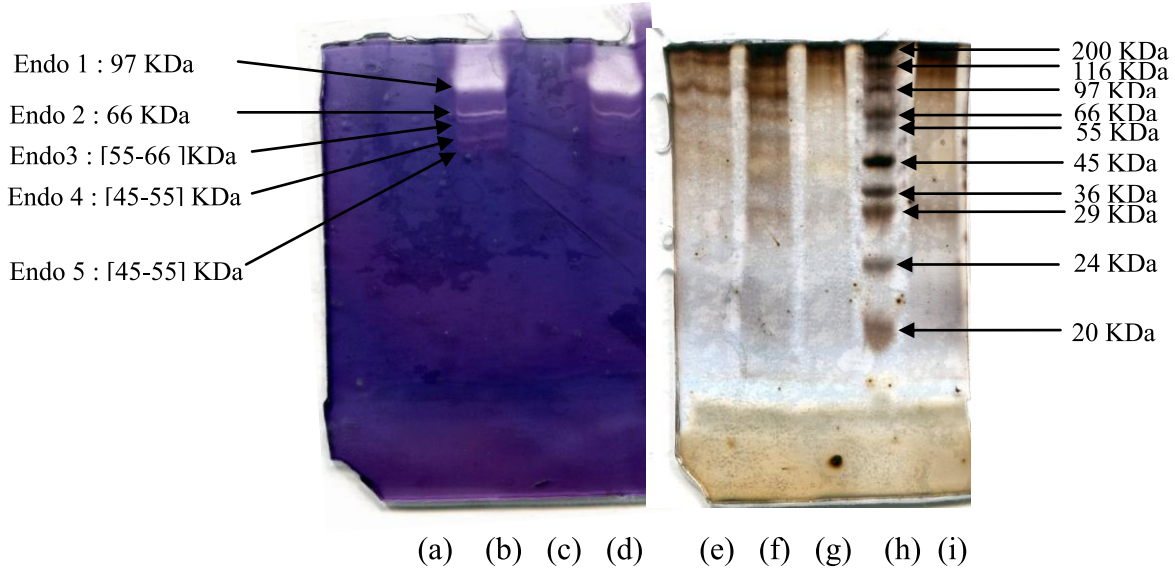


Fig. 7. SDS-PAGE electrophoreses and cellulase zymogram. (a): zymogram of supernatant E4 unheated, (b): zymogram of supernatant E4 with heating (c): zymogram of supernatant E3 unheated (d): zymogram of supernatant E3 with heating. (e): protein profile of E4 from the supernatant without heating (f): protein profile of E4 from the supernatant with heating (g): protein profile of E3 supernatant without heating (h): SIGMA markers, Wide Range, Ref 58445 , (i): protein profile of T3 supernatant with heating.

Table 1. Effect of metal ions and reagents on the activity of the endoglucanase produced by *Streptomyces* sp. B-PNG23.

Metal ions and chemical reagents	Relative activity (%)	Metal ions and chemical reagents	Relative activity (%)
none	100	Na+2	91.60
Ca2+	115.09	Fe2+	135.40
Mg2+	97.79	Sr+2	100
Zn2+	110.6	Ni2+	113.28
NH4+	149.42	Co+2	118
Hg2+	107	PMSF	107
Cu2+	152.75	DTT	81.16
K+	95.93	SDS	101
Mn2+	306.25	EDTA	88.53

activity at 50°C; however, activity decreased sharply beyond this temperature (Li et al., 2008), suggesting that the cellulase secreted by this strain was highly sensitive to changes in temperature. In comparison, our data on pH and temperature showed that the CMCases secreted by B-PNG23 were highly resistant to changes in pH and temperature, and hence well suited to the harsh process conditions that lignocellulose bioprocessing entails. In the same report, Li et al. (2008) stated that *Bacillus* sp. isolated from hot springs has been shown to produce thermostable cellulases; however, the cellulase secreted by this bacterium lost 30% activity within 30 min of incubation at 70°C.

The study of the influence of metal ions on cellulase activity is very important for industrial applications. They have been reported to influence enzyme production by increasing their activity in microorganisms (Rani et al., 2004; Kotchoni et al., 2006). The results of the effect of metal ions and reagents on the activity of endoglucanases are given in Table 1. Results showed that the Mn^{+2} ion strongly stimulates endoglucanase activity with a relative activity of 306.25%; it is assumed that this ion responds to certain amino acid residues in the active site of the protein, causing a conformational change in favor of higher activity. Na^{+2} inhibited endoglucanase activity causing a decrease in the activity of 8.4%. Li et al. (2006) reported the inhibition of endoglucanase from *Bacillus* sp. AC-1 by Fe^{+2} and Cu^{+2}, while the strain B-PNG23 was not affected by Fe^{+2} or Cu^{+2}; similarly the Hg^{+2} ion does not affect negatively endoglucanase activity. The endoglucanases of *Streptomyces* sp B-PNG23 seem resistant to SDS, while the endoglucanase activity was reduced by DTT and EDTA.

B-PNG 23 can secrete many kinds of proteins into the medium. Five different endoglucanases were detected by zymogram analysis, named: Endo1, Endo2, Endo3, Endo4, Endo5, with molecular weights of 97 KDa, 66 Kda, 62.5 KDa, 53 KDa and 50 Kda, respectively. Similarly, *Trichoderma reesei* is also known to produce five different endoglucanases (Karlsson, 2000). Miettinen-Oinonen et al. (2004) reported two endoglucanases produced by the isolate M. *albomyces* ALKO 4237 with molecular weights of 50 and 20 Kda.

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