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PURIFICATION AND CHARACTERIZATION OF AN ENDOGLUCANASE PRODUCED FROM *Streptomyces* sp. STRAINBPNG23

Azzeddine Bettache[*1,2], *Estelle Copinet*[2], *Zahra Azzouz*[1], *Nawel Boucherba*[1], *Cilia Bouiche*[1], *Samir Hamma*[1], *Rima Maibeche*[1], *Francis Duchiron*[2], *Said Benallaoua*[1]

Address(es): Azzeddine Bettache
[1]Laboratoire de Microbiologie Appliquée, faculté des sciences de la nature et de la vie, université de Bejaia, 06000 Bejaia, Algéria.
[2]UMR FARE 614 INRA/URCA. Laboratoire de Microbiologie Industrielle, UFR Sciences, université de Reims Champagne-Ardenne, Moulin de la Housse, 51687 Reims, France.

*Corresponding author: bettache84@hotmail.fr

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ABSTRACT

Streptomyces sp. strain BPNG23 produces five endoglucanases: endo1, endo 2, endo 3, endo 4 and endo 5.The endo2 has been purified and characterized by two subsequent purification steps with ultrafiltration and anion exchange chromatography. The specific activity of the endoglucanase has been found to be 380.65 U/mg. The molecular weight to the endoglucanase 2 has been estimated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis, revealing that this isoenzyme is a 66 KDa monomeric enzyme. It showed an optimum temperature and pH values respectively of 6.0 and 50 °C. It was thermostable, it exhibited a half-life time 6 h with a temperature of 50 °C, the enzyme was activated by several metal ions Mn^{+2}, NH^{4+}, Zn^{2+}, Ca^{2+}, Fe^{2+}, Ni^{2+} and Co^{2+}. It presents a higher affinity towards carboxymethyl cellulose (CMC) with a K_m of 6.37 mg/mL and Vmax of 0.056 µmol/mn. This the first of a study of purification and characterization of an endoglucanase produced by a newly isolated actinobacteria strain in Kabylia region (Algeria).

Keywords: *Streptomyces*, endoglucanase, purification, characterization, wheat bran

INTRODUCTION

Cellulose is one of the most abundant organically produced compounds available in the world and is a kind of sustainable energy which human beings are able to easily utilize **(Bataillon *et al*., 2000)**. The previous research studies have shown the occurrence of several various bacterial and fungal cellulases which can be classed in three different types: endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) **(Battan *et al*., 2007)**. However, a complete degradation of cellulose implies a complex interaction with different cellulolytic enzymes. It was widely agreed and accepted for three types of cellulolytic enzymes which include endoglucanases (EC 3.2.1.4) hydrolyze randomly internal glycosidic linkages in cellulose, whereas exoglucanases cleave long chains of the ends in the progressive chain, β-glucosidases transform cellobiose into glucose **(Beg, 2001)**.
Cellulase is used in bio-fuels biomass fermentation, though the process was relatively experiential in the present. Cellulase has been used for a treatment of phytobezoars, as a form for cellulose bezoar located in the human stomach **(Béguin and Aubert, 1994)**. Cellulases attracted attention with their various applications in both medical and industrial applications. For example, they have been used in decreasing the high level of serum cholesterol, by improving nutrient quality and digestibility **(Blanco *et al*., 1999)**, in animal feed **(Mandels, 1985)**, in waste/water management **(Biely and Mackenzie, 1986)**, in textile (biodegradable textile and producing washed look of denim, etc.) **(Cavaco-Paulo, 1998; Celestino and Felix, 2006)**, in the pulp and the paper industry, and starch transformation **(Beg, 2001)**, in breach and wine-making and in house keep washing detergents **(Mandels, 1985)**.
Actinobacteria are Gram positive filamentous soil bacteria which as the first producers from a large variety of antibiotics and the important bioactive compounds and enzymes, among them major industrial enzymes implicated in lignocelluloses conversion **(Blanco *et al*., 1999)**. The degradation of cellulose, hemicelluloses and lignin, that are abundant of plants, various strains from the Streptomyces genus were studied, and were found as good cellulase producers **(Budihal and Patil, 2016; Shaik *et al*., 2017; Wu *et al*., 2017; Gobalakrishnan and Sivakumar, 2017; Schrempf, 2017; Kumarand Henehan, 2017)**.
Commercial production and efficient utilization of cellulases are infested with many problems. For increasing the efficiency of the process, we could search of a more active cellulase or developing low-cost methods of pretreatment for the substrates, by making more accessible them suitable for enzymatic degradation.
Thus, to mitigate of this problem, the purpose of the present study has been purified and characterized an endoglucanase producing by *Streptomyces* sp. strain BPNG23.

MATERIAL AND METHODS

Microbial strain

Strain BPNG23 was isolated from forest soil of Bejaia in Kabylia region (Algeria). The strain was selected for its potential to produce endoglucanase enzymes. The 16S rRNA gene sequence of the strain BPNG23 has been deposited in GenBank *Streptomyces* under accession number "JQ678705".

Preparation of extracellular crude enzyme

For the cellulases production, 3% pre-culture of *Streptomyces* sp. strain BPNG23 was cultured in medium based on wheat bran: yeast extract (10 g/L), NH_4Cl (2.5 g/L), $MgSO_4$ (0.4 g/L), NaCl (2 g/L) and 10 g/L of wheat bran. The pH was adjusted to 7 and the incubation was performed at 28°C at under agitation rate of 100 rpm, finally the fermentation broth was centrifuged at 10,000×*g* for 10 minute at 4°C.

Purification of the enzyme

The supernatant culture contains secreted cellulases, was concentrated using Minimate PALL system, (France, 10KDa cut-off membrane Millipore, 20 cm x 3.8 cm x 1.8 cm). The concentrated crude enzyme (2 mL) was applied to AKTA Explorer 10 (England) equipped with a strong anion-exchange column Q Sepharose (Hitrap, 5mL) equilibrated with 50 mM Tris–HCl buffer (pH 8.5). Protein was eluted using NaCl gradient (0–1M) in 50 mM Tris–HCl buffer (pH 8.5) at a flow rate of 4 mL/min. A total of number fractions (2mL in each test tube) have been collected and assessed by enzyme activity with protein quantification.

Analytical methods

SDS-PAGE and zymogram analysis of endoglucanase

The crude enzyme (25 µg) in the sample treatment buffer has been boiled for 5 min and was submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis utilizing 10% polyacrylamide. Electrophoresis was carried out using Mini-Gel Electrophoresis unit (Biorad, France).This analysis was performed by Laemmli method, 1970. After the electrophoresis, 0.1% of CMC has been incorporated into running polyacrylamid gel, on completion by electrophoresis; the gel has been cut into two parts. One part has been utilised for Coomassie brilliant blue R-250 staining and the other has been utilised for zymography. This analysis was performed using the basic protocol; the gel was soaked in triton X-100 at 2.5% (w/w) for 30 min, and then incubated in the phosphate buffer 50 mM (pH 7.0) for 30 min at 50°C. The gel was reavaled using 0.1% Congo red solution for 15 min at room temperature (15°C), thereafter washing 1M NaCl and inserted in 0.5% acetic acid for exposing the endoglucanase active bands that contrasts to the dark background.

Enzymatic assay and protein quantification

The supernatant of the fermentation broth has been used for determining the endoglucanase activity; it was assayed using 20g/L (w/v) carboxymethyl cellulose such as the substance that has been suspended in a 50 mM sodium phosphate buffer (pH 7.0). The reaction mixture has been compounded of 250 µl substrate and 250 µl of crude enzyme. The reaction mixture has been incubated at 50°C during 30 min. The releasing reducing sugar has been measured using the 3,5- dinitrosalicylic acid (DNS). The reducing sugar liberated has been measured at 540 nm, utilizing a standard curve with glucose absorbance generated as the standard, in which one unit (U) of endoglucanase activity has been defined as the amount of enzyme that Releases 1 µmol glucose/min/mL in the above conditions. Protein has been quantified using Bradford method **(Bradford, 1976)**, utilizing bovine serum albumin as the standard. Enzymatic activity and protein assays have been performed in triplicate.

Characterization of the enzyme

Molecular weight estimation

The molecular weight for the purified protein has been evaluated using the method of **Laemmli, 1970** employing sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Effect of pH and temperature

The endoglucanase activity has been evaluated at various temperatures (ranging from 30-70°C) and pH values (pH 4-5, sodium acetate; pH 6-7, sodium phosphate pH 8-9 and 10, Tris-HCl) under the method previously described.

Thermostability

Thermostability of endoglucanase has been established by incubating the samples with substrate in a 50 mM sodium phosphate buffer (pH 6.0) from temperatures of 40, 50 and 60°C with an interlude period between 1 and 10 hours. The residual endoglucanase has then been determined by the DNS method.

Substrate specificities

The substrate specificities for the endoglucanase purified were established with the following substrates: 20g/Lbirchwoodxylan, 20g/Lavicel, 20g/L cellobiose and 20 g/L carboxymethyl cellulose prepared in 50 mM sodium phosphate buffer (pH 7.0).

Effect of metal ions and reagents on endoglucanase activity

For the investigation the effects of diverse metal ions on endoglucanase activity, the solutions with 5 mM of Na+, Mg2+, Ca2+, Mn2+, Fe2+, Zn^{2+}, Cu^{2+}, NH^{4+}, Hg^{2+}, K+, Ni^{2+}, Sr2+ and Co^{2+} were additioned in the reaction mixtures which contained the purified endoglucanase into 50 mM sodium phosphate buffer (pH 7. 0), the reaction mixture was considered as control without any additive and the activity of endoglucanase was determined to be 100%. Effect of some inhibitors / chelators: ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), dithiothreitol (DTT) and phenylmethanesulfonyl fluoride (PMSF) were also tested.

Kinetic parameters

The effect of carboxymethyl cellulose, varying from 1 to 10 mg/mL on endoglucanase activity was assessed at optimal assay conditions (50°C, pH 6.0). Kinetic parameters were evaluated using linear regression of double-reciprocal plots according to Lineweaver-Burk, and all tests were carried out in triplicate.

RESULTS AND DISCUSSION

Purification of endoglucanase

After 7 days of fermentation, the medium was centrifuged and the activity was measured in the culture supernatant. The endoglucanase activity obtained was 1.2 U/mL, with specific activity of 1.81 U /mg protein. The culture supernatant was concentrated 20-fold by ultrafiltration to obtain a final volume of 50 mL. At this step, impurities having a molecular weight equal or less than 10 kDa were eliminated. This explains the decrease of protein in the culture supernatant from 660 mg to 22.5 mg in the retentate. The specific activity after ultrafiltration was 23.11U /mg protein. This step improved the purity of the enzyme so that its specific activity was 1.81U/mg in crude supernatant. The yield of this purification process was estimated at 43.3% with a purification factor of 12.76.

Anion exchange chromatography

The chromatogram shows seven protein peaks including three with endoglucanase activity for 0.15 M, 0.35 M and 0.6 M of NaCl (figure 1). All proteins were eluted once the NaCl gradient was realized, this, indicate that the protein fractions were retained by the column. The negatively charged proteins are retained on the column while the neutral and positively charged proteins will be eluted first.

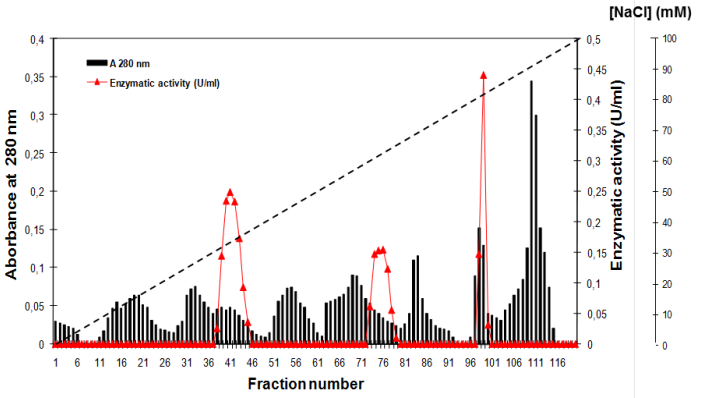

Figure 1 Proteins quantification and elution profil of endoglucanase activity on Qsepharose HI Trap 5mL.

A synthesis for a representative purification procedure was presented in table 1.

Table 1 Purification steps of endoglucanases from *Streptomyces* sp. strain BPNG23

Purification steps	Volume (mL)	Total volume activity (U)	Total proteins (mg)	Specific activity (U/mg)	Yield (%)	Purification Factor
Culture supernatant	1000	1200	660	1.81	100	1
Ultrafiltration	50	520	22.5	23.11	43.3	12.76
Anion exchange chromatography	50	A:12 B: 7.5 C: 19.5	0.037 0.048 0.22	324.32 154 88.6	2.3 1.44 3.75	14.03 6.66 3.83

Anion exchange chromatography was the supreme step in the purification of endoglucanases, the yield decreased respectively to 2.3, 1.44 and 3.75% for the three proteins A, B and C. After each purification step the specific activity of the enzyme increased, the final yield was increased more than 14-fold. It was previously reported than ammonium sulfate precipitation following with anion exchange chromatography gives a purification yield of 9.06 with a B*acillus* sp strain M-9 **(Bajaj *et al.*, 2009)**and 17.5 for *Cellulomonas* sp. YJ5 **(Theberge *et al.*, 1992)**.

Enzyme characterization

Molecular weight

There were five distinct endoglucanases were detected on zymogram analysis, called: Endo1, Endo2, Endo3, Endo4 and Endo5, in which the molecular weights were 97 KDa, 66 KDa, 62.5 KDa, 53 KDa and 50 KDa, respectively, more details are given in our paper **(Bettache *et al.*, 2013)**. The enzyme purified appears of being homogeneous since it migrated in a single band in SDS-PAGE (figure 2).
From the zymogram and the SDS-PAGE profil, it was found of the molecular weight of the endoglucanase A was 66 KDa, which corresponds to endoglucanase 2 found in the concentrated supernatant (figure 2).
Some molecular weights of CMCases produced by various microorganisms were reported: 33 KDa for the CMCase from *Bacillus* sp **(Gupta and Vadehra, 1990)**, 70 kDa in *Neurospora crassa* **(Yazdi *et al.*, 1990)**,the endoglucanases obtained from *Trichoderma viride* have molecular weights of 38, 42, 52 and 60 kDa **(Kim *et al.*, 1994)**, 38 kDa for *Thermomonospora* **(George and Rao, 2001)**, 38 and 74 kDa for *Anabaena laxa* **(Gupta *et al.*, 2012)**.

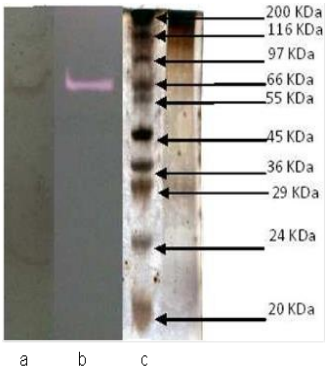

Figure 2 Migration profile of endoglucanase A (SDS-PAGE) and zymogram (a: protein profil; b: Zymogram, c: SIGMA markers)

Effect of pH and temperature on enzyme activity

The purified endoglucanase showed good stability at pH 5.0-8.0, it posted optimum activity a pH 6 (figure 3b) and only 27.68% relative activity a pH 9.The optimum temperature for this endoglucanase activity as shown in Figure 3b at 50°C.
Depending on the optimum temperature, enzymes can be classified such as mesophiles (40-60°C), thermophilic (50-80°C) and hyperthermophilic (>80°C) **(Maheshwari and Bhat, 2000)**, most endoglucanases were stable between 50-55°C **(Lucas *et al.*, 2001)**.The optimum CMCase temperature produced by *Streptomyces* sp. strain B-PNG23 was similar to those produced by *B. amyloliquefaciens* DL-3 **(Lee *et al.*, 2008)**. The thermophilic endoglucanases with optimal activity at 80°C were often reported by thermophilic microorganisms such as *Fervidobacterium Nodosum* **(Wang *et al.*, 2010)**, and *Acidothermus cellulolyticus* **(Lindenmuth and Mcdonald, 2011)**.
The effect of pH on the endoglucanase activity of *Streptomyces* sp. strain BPNG23 was evaluated at 50°C in a buffer system. The optimum pH of the endoglucanase activity of the purified enzyme was 6. In general, endoglucanases are not active at low pH **(Wang *et al.* 2010; Yu, 2012)**.The endoglucanase produced by *Streptomyces* sp. strain BPNG23 was active in a wide pH range, which proves to be an advantage for use under different reaction conditions or biotechnology processes.

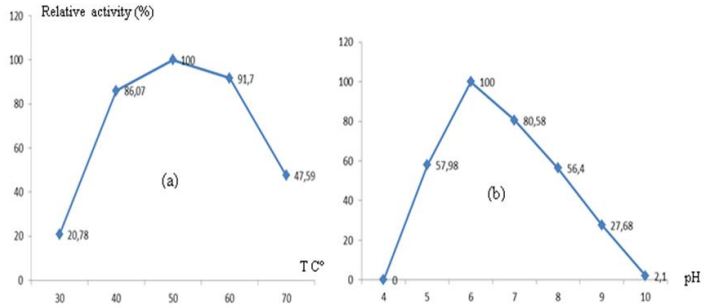

Figure 3 Effect of temperature (a) and pH (b) on the activity of endoglucanase A

Thermostability

At 40°C, the activity was stable it was not significant decrease of endoglucanase activity for 4 h. This enzyme showed a good stability at 50°C, with a half-life of 6 h. At 60°C, there was a sharp drop in relative activity (7.65%); after 4 h of incubation, the relative activity decreased dramatically (figure 4). The endoglucanases of *Streptomes* sp. strain BPNG23 exhibit similar optimal temperatures to those described for*Fusarium oxysporum* (50 and 55°C) **(Christakopoulos, 1995)**, but with higher thermal stability.

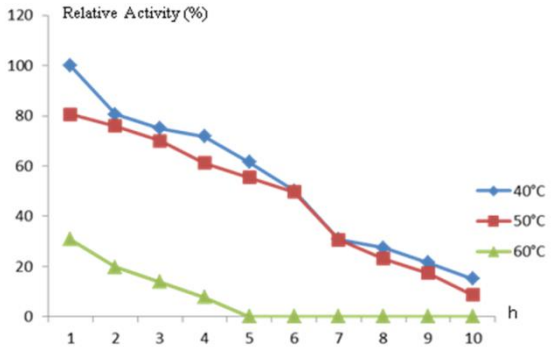

Figure 4 Thermostability of endoglucanase A in the presence of substrate

Substrate specificity

The results show that the purified endoglucanase has more affinity for CMC with a relative activity of 100%. It also degrades cellobiose with a relative activity of 15.35%. For avicel and birch xylan, endoglucanase has no relative activity (figure 5). The use of a variety of polysaccharides checks that the enzyme purified in the present study was an endoglucanase. The "endo" nature of the endoglucanase was confirmed by its high activity on carboxymethyl cellulose and no hydrolysis of the crystalline cellulose **(Béguin and Aubert, 1994)**. *Trichodermareesei*endoglucanase also revealed hydrolytic activity on a wide range of substrates, namely β-glucan, carboxymethyl cellulose, hydroxyethylcellulose, xylan, methylumbelliferyl-β-D-cellobioside and methylumbelliferyl-β -D-lactoside**(Domain *et al.*, 1998)**.

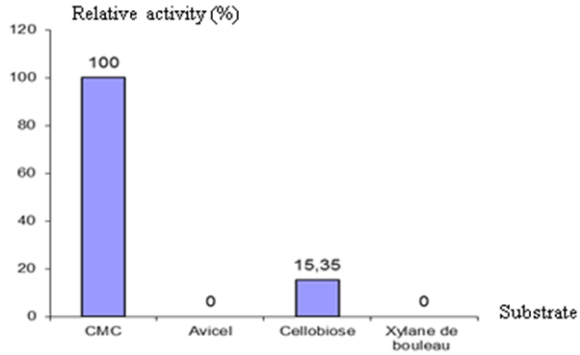

Figure 5 Substrate specificity of endoglucanase A.

Effect of metal ions and reagents on activity

The endoglucanase from *Streptomyces* sp. strain BPNG23 was strongly stimulated by manganese (Mn^{2+}) with a relative activity of 305.4%. The enzyme was also stimulated by Cu^{+2}, NH^{+4}, Zn^{+2}, Ca^{+2}, Fe^{+2}, Ni^{+2} and Co^{+2}, this may be due to the stabilization of the structure of the enzyme (table 2). Higher activity in the presence of Mn^{+2} and Co^{+2} ions was observed in the studies for*Anabaena laxa*endoglucanase**(Gupta *et al.*, 2012)** and endoglucanase of *Daldiniae schscholzii* **(Karnchanatat *et al.*, 2008)**. However, an inhibitory effect by these two ions was observed for the endoglucanases of *Penicillium purpurogenum* **(Lee *et al.*, 2008)** and *Bacillus* sp. **(Yu, 2012)**.
The purified endoglucanase was inhibited by SDS, EDTA, and DTT. Inhibition by EDTA suggests that the enzyme was a metalloprotein. In addition, DTT was a purified endoglucanase inhibitor, suggesting that disulfide bridges are essential for enzymatic activity. The lack of inhibition by PMSF suggests that the serine residue does not play an important role in catalytic activity **(Annamalai and Elayaraja, 2013)**.

Table 2 Effect of metal ions and chemical reagents on the activity of endoglucanase A

Metal ions and chemical reagents	Relative activity (%)	Metal ions and chemical reagents	Relative activity (%)
No	100	Na^{2+}	90.67
Ca^{2+}	115.09	Fe^{2+}	136.02
Mg^{2+}	97.79	Sr^{2+}	99.87
Zn^{2+}	110.6	Ni^{2+}	111. 83
NH^{4+}	149.42	Co^{2+}	117.14
Hg^{2+}	107	PMSF	105.68
Cu^{2+}	152.75	DTT	80.7
K^{+}	96.83	SDS	86.55
Mn^{2+}	305.4	EDTA	87. 36

Kinetic parameters

The Km value is an amount from substrate necessary to achieve the half of initial maximum speed of enzyme; it is also a measurement from the apparently affinity from enzyme of its substrate.

The endoglucanase activity of the *Streptomyces* sp. strain BPNG23 was strongly influenced by the substrate concentration.

A Lineweaver-Burk plot for the activity indicated that the km value of the enzyme to carboxymethyl cellulose was 6.37 mg mL^{-1}; V_{max} of this enzyme was 0.056 µmol mn^{-1}. The endoglucanase with a lower K_m for carboxymethyl cellulose indicates a greater affinity for this substrate.

The K_m values of endoglucanase reported were 3.6 mg mL^{-1} for *Pseudomonas fluorescens* **(Bakare *et al*., 2005)**, 4.97 mg mL^{-1} for the *Actinobacteria anitratus* strain, and 7.90 mg mL^{-1} for *Branhamella* sp **(Ekperigin, 2007)**. **Theberge *et al*., 1992** reported that the endoglucanase of *Streptomyces lividans* has a V_{max} of 24.9 U mg and K_m of 4.2 mg mL^{-1}.

CONCLUSION

Streptomyces sp. strain B-PNG23 was selected for its important productions of endoglucanases. Purification of the endoglucanase of the B-PNG23 strain needed three steps of purification: ultrafiltration, which gives a specific activity of 23.11 and a purification factor of 12.76; anion exchange chromatography which was the main step in the purification procedure, three endoglucanases were detected and eluted at different NaCl concentrations, at this phase the purification yield was greater than 14; molecular sieving which followed the anion exchange chromatography step allowed to increase the specific activity (380.65 U/mg), thus eliminating a very large part of protein contaminants. The enzyme purified was found to be active in a broad range of pH and temperature, which suggests that the enzyme from this strain could be used in the bioconversion to fermentable sugars of lignocellulosic biomass.

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