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Research Article

First Report of Endoglucanase from *Planococcus rifitoensis* Strain M2-26

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Abstract

Here we firstly describe endoglucanase production and biochemical characterization from the moderately halophilic bacterium *Planococcus rifitoensis* strain M2-26. Optimal production has been obtained in the presence of xylose, tryptone and 15% NaCl (w/v). Halotolerant and thermotolerant endoglucanase was produced by *Planococcus rifitoensis* strain M2-26. The enzyme had an optimum activity from 0% to 30% NaCl and at extreme pH value (from 5 to 12) and displayed optimum activity at 110°C. The enzyme was able to retain more than 80% from 20 to 110°C. Endoglucanase activity was increased by Na_2SO_3 and $MnCl_2$ but Hg^{2+} ions have a high inhibitory effect on enzyme activity. The enzyme seems to be a metalloprotein due to the high inhibition obtained by PMSF and EDTA. The enzyme showed a single band on SDS-PAGE with its molecular mass of 85 kDa.

This novel endoglucanase showed excellent activity and stability at extreme pH, salinity and temperature, suggesting its potential use in area industry which required extreme conditions of applications. Because of its activity on corn, barley and straw, the application of the enzyme in the animal food industry was encouraged.

Keywords: Endoglucanase; Planococcus rifitoensis; Thermotolerant Thermostable; Halotolerant; Extreme pH

Abbreviations

DNS: 3,5-Dinitrosalicylic Acid; CMC: Carboxymethylcellulose

Introduction

Halophilic Bacteria produce industrially valuable compounds such as osmoregulants, enzymes, polymers, etc. and they possess a number of interesting applications as well. Because of the extreme nature of enzymes they can execute the current requirement of industry. Extremozymes have a great economic potential in many industrial processes, including agricultural, chemical and pharmaceutical applications [1]. Many consumer products will increasingly benefit from the addition or exploitation of extremozymes. Most of the halophiles produce extracellular hydrolytic enzymes such as amylases, proteases, lipases, DNases, pullulanases and xylanases which have quite diverse potential usage in different areas such as detergent industry, food industry, feed additive, biomedical sciences and chemical industries [2].

Cellulose is an abundant natural biopolymer on earth and most dominating agricultural waste. This cellulosic biomass is a renewable and abundant resource with great potential for bioconversion to value-added Bioproducts. It can be degraded by cellulase produced by cellulolytic bacteria. Cellulases are classified into three groups: exoglucanases, endoglucanases and β–D–glucosidases. Exoglucanases cleave the cellobiosyl units from the non-reducing ends of the cellulose chains. Endoglucanases hydrolyze the internal cellulosic linkages and β–D–glucosidases specifically cleave glucosyl units from the non-reducing ends of cello-oligosaccharides. Endoglucanases cut at random at internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides of various lengths and consequently new chain ends. It is generally active against acid-swollen amorphous cellulose, soluble derivatives of cellulose such as CMC, cello oligosaccharides [3].

Cellulases are used in textile industries, in detergent industries and bioremediation of cellulosic waste [4]. Now a day, cellulases are widely used in the fermentation industries for converting cellulose to fermentable sugar [5]. These industrial applications necessitate endoglucanases to be sufficiently robust and stable under extremes conditions of intended industrial applications. Hence, obtaining endoglucanases with new physiochemical properties is an important endeavor. However, halophilic cellulase can be derived from bacteria such as *Bacillus sp* [7], *Salinivibrio sp* [5], limited reports are available for halophilic cellulases from moderately halophilic and halotolerant bacteria, and there is no report describing cellulase from the moderately halophilic bacteria specie *Planococcus rifitoensis*. In this work, we used the strain M2-26 of *Planococcus rifitoensis* previously isolated from Tunisian sebkha and identified as a good agent of biocontrol by producing antifungal enzymes such as Chitinase [8]. Here we firstly report the production of endoglucanase by *Planococcus rifitoensis* strain M2-26, and describe the partial purification and characterization of the enzyme.

Materials and Methods

Microorganisms and culture condition

A new moderately halophilic bacterium *Planococcus rifitoensis* strain M2-26 was isolated from a shallow salt lake in Tunisia. The nucle¬otide sequence of 16S rRNA was previously reported and has been deposited in the GenBank database under the accession number EF471920 [9]. The strain M2-26 of *P. rifitoensis*, was carried out in a medium containing: 5 g/L Tryptone: 1 g/L cellulose: 5 g/L YE, 1g/L K_2HPO_4 and NaCl: 5g/L, pH = 7.2. Bacterial cells (100 µl) from a 48h fresh culture on Tryptic Soy broth (Difco, USA) were inoculated and incubated at 37°C for 5 days on a rotary shaker (120 rpm). After centrifugation at 12000 rpm for 10 min, the supernatant were collected for endoglucanase assay with three independent replications. The effect of salinity on enzyme was evalu-

ated by growing the bacterium in the same medium supplemented with a gradient of salt (0-5-10-15-20-25 and 30% NaCl, w/v), at 37°C for 5 days.

Endoglucanase assay

Cellulase activity was measured by DNS assay according to the method of [10]. The mixture (v/v) reaction was composed of an enzyme sample (cell free supernatant), 1% CMC and citrate buffer, and it was incubated at 50°C for 30 minutes, after that 800 ml of DNS was added to stop reaction. The product was determined by 3, 5-dinitrosalicylic acid assay (DNS), and the absorbance was measured at 540 nm. One unit (U) of endoglucanase activity was defined as the amount of enzyme that liberated 1 mmol of glucose in one min [11].

Effect of salts on enzyme production

The same medium was supplemented with 15% of NaCl, KCl, $NaNO_3$, sodium acetate (C_2H_3O2Na), citrate sodium ($Na_3C_6H_5O_7$), ammonium nitrate (NH_4NO_3) and sodium sulphate (Na2SO4). Cultures were incubated for 48h at 37°C on a rotary shaker (120 rev min-1).

Effect of carbon source on enzyme production

The same medium composition cited above was used but the source of carbon (cellulose) was changed by a list of carbon sources (xylan, dextrin, maltose, mannose, xylose, saccharose, galactose, raffinose, fructose, lactose, mannitol, carboxymethyl-cellulose, α-cellulose) was additioned separately at 0,1% 5 and the filter paper, corn, straw and barley At 1%, 2% 3% or 4% (w/v) [12].

Effect of nitrogen source on endoglucanase production

Yeast extract in the medium was replaced by various nitrogen sources organic and inorganic at 0.5%. The carbon source used was xylose at 0.1% with 15% NaCl. Triplicate samples were removed after 2 days of growth on a rotary shaker 120 rev min-1 at 37°C [13].

Temperature optima of enzyme

The reaction mixture was incubated at different temperatures from 20 to 120°C. Thermal stability was examined by pre-incubation of the enzyme for 30 minutes at various temperatures, the residual activity of the enzyme was then determined at 110°C [14].

Effect of pH on endoglucanase activity and stability

The substrate emulsion was prepared in various buffers with varying pH (4 to 13). The optimal pH for enzyme activity was measured at different pH values. The buffers used were as fol¬lows (0.05 M): tampon sodium citrate buffer, pH 4,0 - 5,0), sodium phosphate buffer (pH 6,0 - 7,5), Tris-HCl (pH 8,0 -10,0), and Na_2HPO_4 -NaOH buffer (pH 11,0 -13,0), incubation reaction at 110°C for 30 minutes. The residual activity of the enzyme was examined by incubating the enzyme solution in Buffers cited above. After incubation of reaction mixture for 24h, the residual activity was measured at 110°C and pH 5 for 30 minutes [15].

Partial Purification of endoglucanase

The crude endoglucanase was fractioned by ammonium sulphate precipitation. Various fractions were collected, i.e. 20%, 33.33%, 50%. 60%, 66.66%, 71.4% and 75%. Precipitated fractions were collected by centrifugation of broth at 10,000 rpm at 4°C for 10 minutes. The fractions were dissolved in a little amount of phosphate buffer and dialyzed by a dialysis membrane (Himedia) at 4°C for overnight. Dialyzed enzyme was used as a source of crude enzyme.

Polyacrylamide gel electrophoresis and zymogram analysis

Native PAGE was performed at 4°C with a 10% polyacrylamide gel according to the Laemmli method [16] by using BioRad Mini Protean II apparatus in 100v. The gel was stained with Coomassie blue (R250). The SDS–PAGE was performed with 12% polyacrylamide gel. The molecular mass of the subunits was estimated with standard markers (BioRad Rang protein Molecular Weight Markers 200 kDa, Promega). After electrophoresis, one part of the gel was stained with Coomassie blue and the other one was washed twice, and incubated for at least 3h in 1% CMC solution, after that the gel was rinsing with demineralized water and bands were detected by the addition of 0.1% Congo red for 1h.

Results and Discussion

Few reports investigate cellulases from halophilic bacteria so that further research was of great importance of cellulase from halophilic bacteria. Different form of cellulose used to detect cellulase enzymes, but carboxymethylcellulose (CMC) was the soluble form of cellulose used as the excellent substrate of endoglucanase and the majority of bacterial cellulases seem to be endoglucanases [17].

The halotolerant endoglucanase produced by strain M2-26 was able to operating in the absence of NaCl and in the presence of high salinity 30% NaCl (w/v) with the maximum production at 15% NaCl (w/v).The maximum enzymes production by the strain M2-26 was obtained after incubation for 2 days at 37°C. The effect salt results show that the addition of KCl or Sodium Sulfate was able to retain 94.2% of enzyme activity at tested conditions respectively 88% and 85% in the presence of Sodium nitrate, Sodium citrate, unlike the enzyme were only able to retain 30% of its activity in the absence of any salt (Table 1).

Salt 15% (w/v)	Growth	Endoglucanase activity (%)
KCl	+	94.2 ± 1.13b
Sodium Acetate	+	79.2 ± 1.3c
Sodium Sulphate	+	94.2 ± 1.13b
Ammonium Nitrate	+	79.2 ± 1.6c
Sodium Citrate	+	85 ± 1.25bc
NaCl	+	100 ± 0.3a
Sodium Nitrate	+	88 ± 1.26bc
Control	+	30 ± 1.14d

Table 1: Nature salt effect at 15% (w/v) on endoglucanase production by strain M2-26 of P. rifitoensis.

These data are in agreement with other works concerning the halotolerance of endoglucanases produced by halophilic bacteria such as: *Salinivibrio sp.* NTU-05 [5], *Bacillus flexus* [14] and *Bacillus licheniformis* C108 [16]. Moreover, on industrial area, it was with great importance to have enzyme active at extreme salt conditions (0% and 30% NaCl), For example paper industry, sauce soja [14]. Similar to others halophilic species, the strain M2-26 take optimum at 15% NaCl [14,19]. Maximum production after 48h, at stationary phase, compared to other works, when endoglucanase production was obtained after in accelerated phase 50h for *Clostridium cellulolyticum* [20], 96h for *Thermomonospora fusca* [21] and 72h for *Bacillus flexus* [14].

Substrates	Substrates Concentration			
Carbon	1%	2%	3%	4%
Filter Paper	1,5 ± 0	1,3 ± 0	1,5 ± 0	1,3 ± 0
Straw	1,8 ± 0	2,14 ± 0	2,6 ± 0	2,62 ± 0
Barley	1,6 ± 0	2 ± 0	2,5 ± 0,2	1,3 ± 0,1
Corn	1,1 ± 0	1,2 ± 0	1,9 ± 0	2,13 ± 0

Table 2: Effect of endoglucanase on agriculture waste.

The most inductor carbon source of the enzyme production was Xylose than, Galactose and Mannose respectively, with rela-

tive activities of 100%, 87.4% and 78.5%. Unlike, in the presence of all other tested carbon source enzyme production doesn't exceed 52.94% (Figure 1).

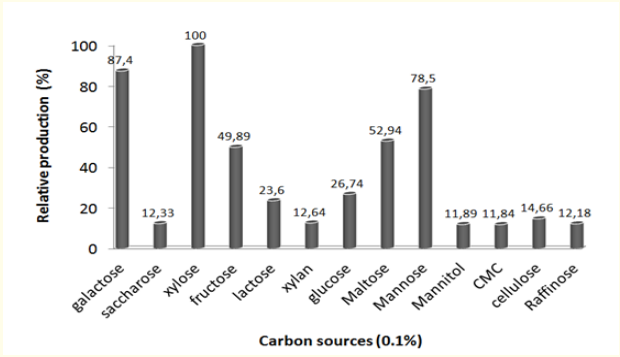

Figure 1: Carbon sources effect on endoglucanase production in the presence of 15% NaCl (w/v).

This work was the first to demonstrate the induction effect of xylose as carbon sources, at the opposite of others study in with cellulose was the major production enzyme inductor by *Bacillus* sp. and *Geobacillus* sp [22]. or CMC by *Bacillus subtilis* A-53 [15]. But *Trichoderma reesei* RUT-C30 give the maximum enzyme activity in the presence of a mixture of cellulose and xylose [23]. Holtzapple., *et al.* 2004, confirm that carbon sources affect the endoglucanase production at stationary phase. In the present work, strain M2-26 has maximum enzyme production in the presence of tryptone (165.74%) than potassium nitrate (110.36%) similar results were given by the work of [12,13]. The addition of tryptone as organic nitrogen sources at 0.5% with yeast extract, has stimulated the enzyme production with 165.74%, compared to the presence of yeast extract only with 100% relative production. Unlike peptone has negligible effect on enzyme production (Table 3). The temperature optimum for enzyme activity was at 110°C and it was able to retain above 90% of its activity in the presence of temperature varying from 20 to 90°C and 80% of the activity was retained at 100 and 110°C (Figure 2A). Various studies reveal the effect of temperature on endoglucanase activity from halophilic bacteria. Besides, the present study was the first to describe the optimum temperature of endoglucanase at 110°C produced by the moderately halophilic bacterium strain M2-26 of *P. rifitoensis.* This value was more important than others published such as: *Salinivibrio* sp. optimum at 35°C [5], *Bacillus flexus* optimum activity at 45°C [14], and *Bacillus* sp. C14 (optimum at 50°C) [4]. Figure 2B shows that pH5 was the optimum at 110°C, 97.7% of activity was given at pH6 and more than 86% of endoglucanase activity was retained at pH values from 7 to 8 and more than 67% from pH value ranging from 9 to 13. Thus, the endoglucanase enzyme from strain M2-26 can be classified as an acidic enzyme. The enzyme was able to retain more than 98% of its activities at pH 5 and 6 and more than 64% of its activities at pH value 4, 7 and 8 but only 52% of activity was retained at pH varying from 9 to 12. The endoglucanase produced by the strain M2-26 has optimal activity at acidic pH (pH5), like some halophilic bacteria possess [15]. But the endoglucanase produced by strain M2-26 described here was also active at pH alkaline so the tolerance of this enzyme at extreme pH. Indeed, the stability and the activity of such enzyme in extreme pH5 and pH12 presents a property which in not only very rare, but which will give an important value for this enzyme to use in industrial applications, which in the majority of the cases require extreme values of pH such as the extraction of the agar from seaweeds (acidic pH), the industry of cleaner (pH alkaline) [28]. Enzyme activity was affected by EDTA, SDS, PMSF, high inhibition was obtained respectively by PMSF and EDTA, with 87.21% and 75.31% but un-important inhibition of about 15.5% of activity was observed by the addition of SDS (Table 4). The apparent molecular mass of native endoglucanase was determined to be about 85 kDa. SDS-PAGE revealed only one subunit, with an apparent molecular mass of 85 kDa. In literature, the molecular weight of the endoglucanase, vary from bacteria specie to other [4,5,15]. Here we have detected one band in NATIVE PAGE, further study was needed to determine the monomeric nature of the endoglucanase described here. Major halophilic endoglucanases earlier published were also monomeric such as *B. sphaericus* JS1 [13] possess endoglucanase with 183 kDa constituted with 4 subunits of 42 kDa.

Nitrogen Sources	Relative Production (%)
0.5% Yeast extract (YE)	100 ± 1.58bc
0.5% YE+ 0.5% Tryptone	165.74 ± 1.62a
0.5% YE+ 0.5% Peptone	102.71 ± 1.27bc
0.5% YE+ 0.5% Ammonium chlorure	100 ± 1.81bc
0.5% YE+ 0.5% Ammonium nitrate	102.67 ± 1.3bc
0.5% YE+ 0.5% Potassium nitrate	110.36 ± 1.54b
0.5% YE+ 0.5% Urea	87.36 ± 1.22c

Table 3: Effect of the nitrogen sources on endoglucanase production by strain M2-26 in the presence of 15 % NaCl, and xylose as carbon source Tryptone, peptone, Ammonium chlorure, Ammonium nitrate, Potassium nitrate and Urea.

Additives Type	Relative Activity (%) (*)
Control	100 ± 1.5d
SDS (1%)	84.5 ± 1.53e
PMSF (5 mM)	12.79 ± 1.24h
EDTA (5 mM)	24.69 ± 1.4g

Table 4: The additives effect on the endoglucanase activity. The enzyme expressed in percentage with test of multiple comparisons of the averages of groups (Test of Duncan).

(*) : values are expressed in percentage. 100% corresponds to the enzymatic activity obtained in the control reaction (in the absence of any additive). The data are the averages of three repetitions ± the standard error. The averages presented by the same letter do not present significant differences (P > 0.05) according to the test ANOA (XLSTAT software).

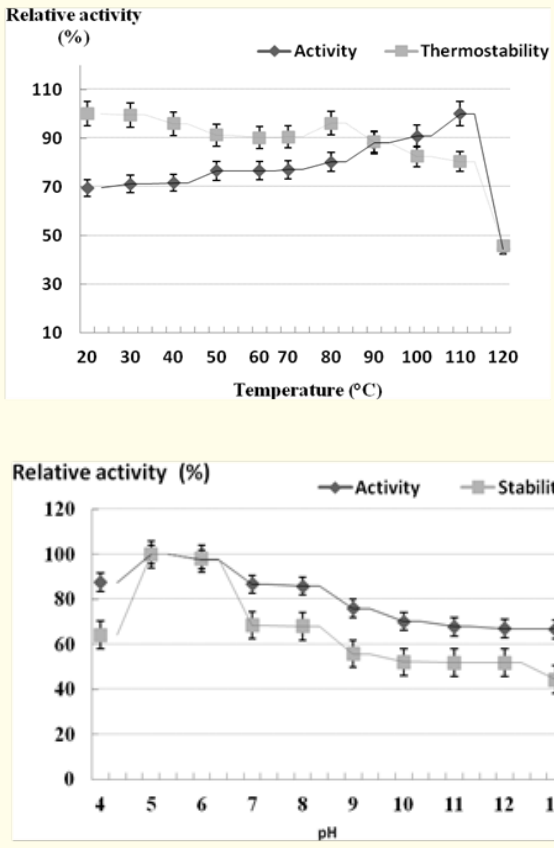

Figure 2: Temperature (A) and pH (B) effect on endoglucanase activity and stability of Planococcus rifitoensis strain M2-26.

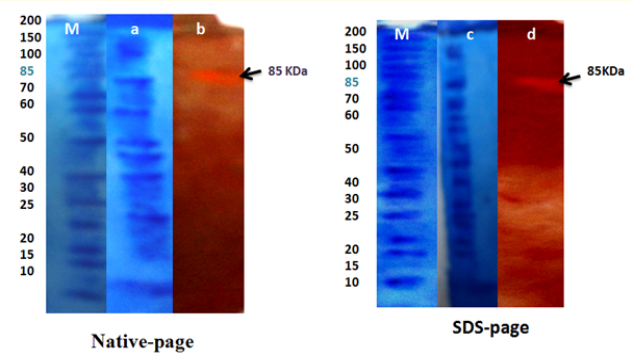

Figure 3: Electrophoresis in non-denaturing polyacrylamide (native PAGE) gels and denatured gel (SDS–PAGE) analysis of the concentrated and dialyzed supernatant from strain M2-26; (a) the concentrated dialyzed supernatant stained with Coomassie Blue. The gel was analyzed for endoglucanase activity by incubating in CM solution in native page (b) and SDS page (d) (c) SDS–PAGE gels analysis of the concentrated and dialysed supernatant from strain M2-26, stained with Coomassie Blue. M: molecular weight protein marker of 225 kDa.

Conclusion

This characteristic of the thermostability and the thermotolerance of the enzyme produced by the strain M2-26 prove that the halophilic bacteria also present an important source of thermostables enzymes which do not exclusively arise from thermophiles species. On the other hand, this property of thermostability and thermotolerance will give an eminent potentiality to apply this enzyme in the industrial domain requiring extreme conditions of temperature such as the paper bleaching. Here the described characteristic of the enzyme resistance to the SDS make its importance to the application in the detergent industry. The wide pH stability (4-7) and temperature stability up to 50°C of endoglucanase makes the enzyme suitable for use in cellulose saccharification at moderate temperature.

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