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Characterization of Cellulases of Thermopiles Bacteria from Rimbo Panti Hot Spring, West Sumatera, Indonesia

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The thermopiles bacteria from hot springs Rimbo Panti, West Sumatera, Indonesia was screened. Screening is done by using a selection medium for thermopiles bacteria and to examine the potential of cellulases used medium containing carboxy methyl cellulose and stained with Congo red which marked the formation of clear zones, where the isolates obtained was 14. In this study, the effect of temperature and pH on endoglucanases activity showed that, it is thermo-tolerant enzyme because at the temperature from 50-100 ºC still show a fairly stable and increas the activity up to 100 ºC, with the tolerant of pH ranging from pH 4.8 to 7.5. The three isolates were also thermo-stable at temperatures 70 to 100 ºC. Maximum activity of exoglucanases produced by the 1M-2 isolates sample is 40.20 units/mg at 70 ºC and pH 6.5, on the other hand, the highest maximum activity of endoglucanases produced by, isolates 1M-3 is 70.50 units/mg, at optimum conditions of temperature 100 ºC and pH 7.5. Moreover, the highest maximum activity of β-glucosidases produced by, 1M-2 isolates is 65.30 units/mg at temperature 80 ºC and pH 7.5.

Key Words: Thermopiles bacteria, Exoglucanases, Endoglucanases, β-Glucosidases.

INTRODUCTION

Celulolytic enzyme is responsible for hydrolytic cleavage of β-glycosidic bond in cellulose polymer to release glucose units[1,2]. They can be broadly classified into three types based on their mode of action: (a) endoglucanases or carboxy-methyl cellulases (CMCases) (endo-1,4-glucanasa, EC. 3.2.1.4), bring out hydrolysis of internal glycosidic bonds in cellulose chains, creating more terminal ends in the fragment, (b) exoglucanases or cellobiohydrolases (exo-1,4-glucanases, EC. 3.2.1.91) liberate glucose or cellobiose from end of cellulose chains/fragment due to hydrolysis of terminal glycosidic bonds and (c) β-1,4-glucosidases (β-D-glucohydrolases, EC. 3.2.1.21) was hydrolysis cellobiose formed as a result of synergic action of the above two enzymes[3-6]. Bacteria, due to their high diversity, faster growth and capability to produce highly thermo-stable enzymes, are ideal to be used in industries as highly potent and robust sources of industrially important enzymes[7]. Degradation of cellulose at elevated temperature provides many benefits, such as increased cellulase activity, lessened energy costs for cooling and decreased risk of contamination[8]. Thermostable cellulase has high-level activity under thermal conditions. Thus, it is an attractive candidate for practical applications[9].

Assays for determining cellulase activity have been classified differently over years of cellulase research. Sharrockc[10] grouped cellulase assays into two basic approaches, namely activities of individual cellulases (EGs, exoglucanases and β-glucosidases) and measuring the total saccharifying activity of a crude cellulase system. Cellulase activity is mainly evaluated using a reducing sugar assay to measure the end products of cellulase hydrolysis activities. More over, the results of such an assay are typically expressed as the hydrolysis capacity of the enzymes. The present work reported the characterization of cellulases of thermopiles bacteria screened from Rimbo Panti Hot Spring, West Sumatera, Indonesia.

EXPERIMENTAL

The materials used for this study were in analytical grade. Carboxyl methyl cellulose (CMC), 0.05 mol/L citrate buffer pH 4.8 Avicell (microcrystalline cellulose), 0.05 mol/L sodium acetate buffer (pH 4.5), glucose, 3.5 di-nitro salicylic acid (DNS) reducing sugar reagent, Glucostat 4X Reagent Set (Worthington Biochemical Corporation, USA), cellobiose, Bio Rad Protein Assay (Commassie Brilliant Blue G-250), $(NH_4)_2SO_4$, phosphate buffer (pH 7.5).

The medium selection for thermopiles bacteria, to the volume of 1 L are: tryptone, yeast extract, $CaCl_2$, galerite gallen gum, agar, vitamin 1 % (v/v) (consisting of pyridoxine 0.01 g, 0.05 g nicotinic acid, thiamine 0.05 g, 0.02 g biotin dissolved in 100 mL of distilled water, filtered with filter 0.2 m) and minerals, 1 % (v/v) (consisting of $MgSO_4 \cdot 7H_2O$ 0.3 g, $MnSO_4 \cdot H_2O$ 12.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, $CoCl_2$ 0.01 g, $ZnSO_4 \cdot 7H_2O$ 0.01 g, $CuSO_4 \cdot 5H_2O$ 0.01 g dissolved in 100 mL distilled water), medium adjusted to pH 8 with $CaCO_3$.

Medium composition for the formation of clear zone as follows for the volume of 1 L: 2 g ammonium sulphate, 2 g KH_2PO_4, 0.3 g urea, 0.3 g $CaCl_2$, NaCl 0.1 g, $FeSO_4 \cdot 7H_2O$ 5 mg, 1.4 mg $MnSO_4 \cdot 7H_2O$, 2 mg $CoCl_2$, CMC 1.5 g, 4 g peptone, 15 g bacto-agar sterilized medium.

High pressure steam sterilizer BS-245, Water Bath-Shaker Taitex. Personal-11, Clean Bench . Hitachi, UV-1600, UV-VIS spectrophotometre Shimadzu, pH Messer model 80 Griffin (Griffin & George Southborough, UK) and glassware commonly used laboratory.

Screening of thermophilic bacteria: Screening performed on water samples from hot springs Rimbo Panti, West Sumatra, Indonesia using the selection medium for thermopiles bacteria, is done by pouring the water sample (5 mL) into Petri, then poured medium selection. In a Petri disc containing the selection medium and hot water sample will grow colonies of thermopiles bacteria after incubation at 70 ºC for 2 days and 50 ºC for 3 days. Each colony was separated and grown on selection medium to obtain single colonies on each Petri's at the same incubation temperature. The separation of the colonies continued until single colony of isolates obtained in each Petri.

Determination of potential cellulase activity: Cellulase activity observed with the formation of clear zone using a medium formation of clear zone, containing carboxy methyl cellulose and with Congo red dye, Cultivate every single isolates into each dish and incubation at 50 ºC for 2 days, measure the diameter of the colony of thermopiles bacteria and then flooding medium surface with Congo red 0.1 % (v/v) left for 5 min and faded with 1 mol/L NaCl, it would seem clear zone around thermopiles bacterial colonies. Halo diameters were measured and compared between all isolates.

Isolation of enzymes: Thermopiles bacteria were grown in liquid medium with the composition of the medium as follows for the volume of 1 L: 2 g ammonium sulphate, 2 g KH_2PO_4, 0.3 g urea, 0.3 g $CaCl_2$, NaCl 0.1 g, $FeSO_4 \cdot 7H_2O$ 5 mg, 1.4 mg $MnSO_4 \cdot 7H_2O$, $CoCl_2$ 2 mg, CMC 1.5 g, 4 g peptone. Medium (100 mL), poured into Erlenmeyer flasks (250 mL), sterilized and inoculated bacterial culture into it and then incubated in a water bath, shaker at 150 rpm with temperature 50 ºC for 48 h. Separate the cells and supernatant in a centrifuge at a speed of 10,000 rpm for 10 min, take supernatant. Supernatant was precipitated with ammonium sulphate (80 % saturation), all steps performed at 4 ºC. The precipitate was re-suspended in phosphate buffer (10 mmol/L), pH 7.5, then dialysis in 3 L of the same buffer for 36 h by replacing the buffer three times every 12 h. Supernatant is to be tested cellulase enzyme activity.

Assay of exoglucanase activity: Activity was measured using the method of Ghosh[11] with commercial Avicell (microcrystalline cellulose) as substrate; avicell suspension (1 %) (w/v) in 0.05 mol/L. Sodium acetate buffer pH 4.5 and using glucose as standard. Exoglucanase enzyme activity assay, using 1 mL of liquid culture containing enzymes exoglucanase put into test tubes (25 mL) add 1 mL of substrate avicell shake with vortex and then incubated for 1 h at 50 ºC in a water bath-shaker. The reaction was stopped with 3 mL of DNS reagent shake with vortex and the test tubes is inserted into the boiling water for 5-10 min then cool in ice water (ice bath) and add 20 mL of distilled water, shake with vortex and measuring with a spectrophotometer at 540 nm.

Assay of endoglucanases activity: Activity was measured using the method of Ghosh[11] is based on measuring the liberated reducing sugars as a result of degradation of cellulose. Substrate used was carboxy methyl cellulose (2 %), dissolved in 0.05 mol/L citrate buffer pH 4.8 and used glucose as standard. Cellulase enzyme activity assay performed using 0.5 mL of liquid culture containing cellulase enzyme is inserted into a test tube (25 mL) was added 0.5 mL carboxy methyl cellulose substrate, shaken by vortex and then incubated for 0.5 h at 50 ºC in a water bath-shaker. The reaction was stopped with 3 mL of DNS reagent shake with vortex and the test tubes is inserted into the boiling water for 5-10 min then cool in ice water (ice bath) and add 20 mL of distilled water, shake with vortex and measuring with a spectrophotometer at 540 nm. Cellulase activity is expressed 1 unit = 1.0 mol glucose released from cellulose per minute (incubation time, 0.5 h and temperature 50 ºC).

Assay of β-glucosidases activity: Activity was measured using the method of Mandel[12] with glucostat 4X reagent set (Worthington Biochemical Corporation) USA and cellobiose as substrate. To prepare the reagents; 1 bottle glucostat, dissolved in 160 mL of distilled water (store in refrigerator) and 1 bottle chromagen dissolved in 160 mL of distilled water (store in refrigerator), mix glucostat and chromagen with a ratio of 1:1 used in fresh condition. Dissolve 20 mmol/L cellobiose (684 mg/100mL) in 0.05 mol/L citrate buffer, pH 4.5 and the standard use of glucose. Assay of β-glucosidase enzyme activity using 1 mL of liquid culture containing β-glucosidase enzyme is inserted into the test tube 25 mL add 1 mL of substrate cellobiose (20 mmol/L) was shaken with a vortex and then incubated for 0.5 h at 50 ºC in a water bath-shaker, then the reaction tube is inserted into the boiling water for 5 min then cool and add 5 mL of reagent glucostat on each tube at intervals of 20 s with a vortex shake allow 10 min, add 4 drops of 5 mol/L HCl shake vortex leave 5 min and measured by spectrophotometer at 420 nm.

Effect of temperature and pH activity against exoglucanase, endoglucanase and β-glucosidase: Effect of temperature on the activity carried out by, incubation temperature variations of 37, 40, 50, 60, 70, 80, 90 and 100 ºC with an incubation time 0.5 h and pH 4.8. Effect of pH on the enzyme activity carried out by variation of pH from 4 to 8 with an incubation time of 0.5 h at 50 ºC.

Determination of cellulases protein: Protein of cellulases was determined by Bio Rad Protein Assay (Commassie Brilliant Blue G-250). The assay is based on the method of Bradford[13]. In this assay BSA was used as a standard, 0.1 mL filtrate enzyme is added 5 mL of Bio Rad reagent mixture with vortex

and then incubated for 5 min at room temperature. Measured by spectrophotometer in 595 nm.

RESULTS AND DISCUSSION

Enzyme activity of cellulase: Activity of exoglucanase, endoglucanase and β-glucosidase obtained from 14 isolates, respectively from 2.74 to 18.23 units/mg for exoglucanase, 6.64 to 58.40 units/mg for endoglucanase, 3.56 to 49.8 units/mg for β-glucosidase at standard conditions of assay, protein content and glucose released from cellulose per minute as shown in Table-1. The result shows that the activity is lower than endoglucanase exoglucanase and β-glucosidase. In Fig. 1, enzymes activity exoglucanase, endoglucanase and β-glucosidase of 14 isolates found 6 isolates that have high activity as compared with pure cellulase from *Aspergillus niger*. Then to the effect of temperature and pH was performed in 6 isolates.

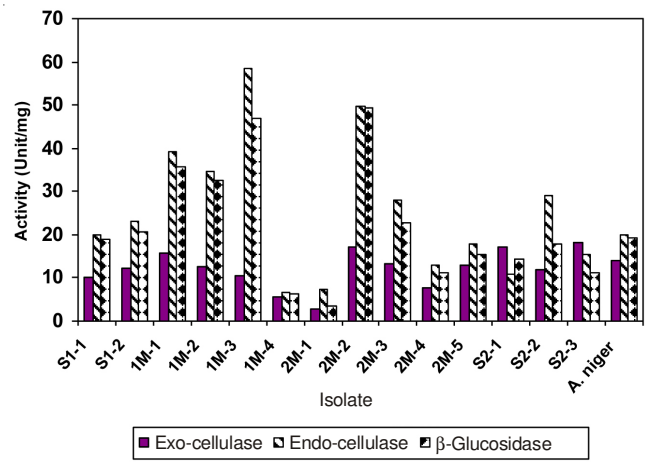

Fig. 1. Activity of, enzymes exoglucanase, endoglucanase and β-glucosidase from 14 isolates of thermophilic bacteria at standard condition, for exo-glucanase (temperature 50 ºC. pH 4.5, acetate buffer, incubation time 1 h), for endoglucanase (temperature 50 ºC, pH 4.8, buffer citrate and incubation time 0.5 h) and for β-glucosidase (temperature 50 ºC, pH 4,5, citrate buffer and incubation time 0.5 h)

Effects of temperature and pH on activity exoglucanases: Effects of temperature and pH on the activity exoglucanase are shows in Fig. 2A-B. As shown in Fig. 2A-B, from isolate thermophilic bacterial, 6 isolates, *i.e.*, shows high activity compared with the cellulase from *Aspergillus niger*, with a maximum activity 35.43, 38.90, 40.20 and 35.05 units/mg, respectively. Temperature and pH optimum for 1M-1 isolates are 80 ºC and pH 7 with maximum activity of 35.08 units/mg. Moreover for 2M-2 isolates the optimum temperature and pH are, 60 ºC and 6.0 with maximum activity of 32.65 units/mg. The maximum activity for exoglucanase produced by the 1M-2 isolates is 40.20 units/mg at optimum conditions. The results shows higher than the pure enzyme from *Aspergillus niger*, with maximum activity of 37.85 units/mg at the optimum temperature 50 ºC and pH 7. The comparation of production formation rate for both exoglucanase and endoglucanase from mutant and parent *Bacillus subtilis* GQ301542 were (41.5 and 65.5 IU/L/h) and (18.50 and 48.00 IU/L/h), respectively.

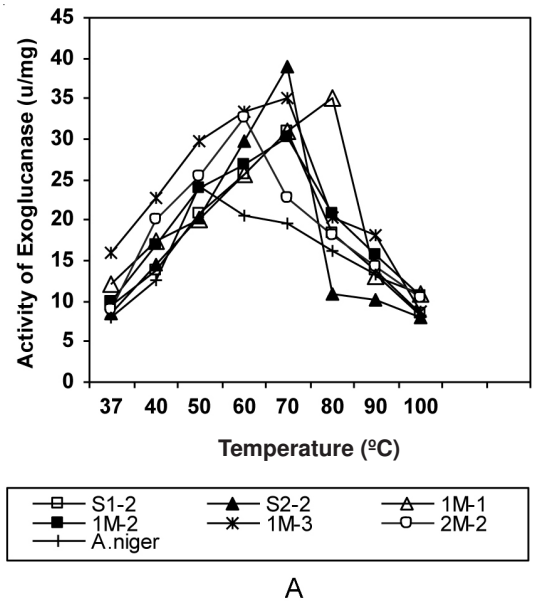

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TABLE-1
PROTEIN CONTENT, THE LIBERATED GLUCOSE, ACTIVITY OF ENDOGLUCANASE, EXOGLUCANASE AND β-GLUCOSIDASE FROM ISOLATES OF THERMOPHILIC BACTERIA

No.	Code isolate	Endoglucanase				Exoglucanase			β-Glucosidase		
		Protein (mg)	Glucose (mg)	Activity		Glucose (mg)	Activity		Glucose (mg)	Activity	
				Unit	U (mg)		Unit	U (mg)		Unit	U (mg)
1	S1-1	0.040	2.150	0.795	19.75	8.984	0.832	20.81	8.099	0.750	18.75
2	S1-2	0.034	2.114	0.782	23.00	9.287	0.860	25.30	7.589	0.703	20.67
3	1M-1	0.013	1.372	0.507	39.49	4.806	0.445	34.24	5.000	0.463	35.65
4	1M-2	0.021	1.970	0.729	34.14	6.792	0.629	29.95	9.330	0.864	32.59
5	1M-3	0.015	2.368	0.876	58.40	7.192	0.666	44.40	7.591	0.703	46.86
6	1M-4	0.110	2.004	0.731	6.64	6.501	0.602	5.46	7.289	0.675	6.13
7	2M-1	0.109	2.172	0.804	7.3 7	3.228	0.299	2.74	4.190	0.388	3.56
8	2M-2	0.009	1.210	0.447	49.66	3.596	0.333	37.00	4.794	0.444	49.38
9	2M-3	0.009	1.664	0.615	27.95	3.196	0.296	13.45	4.848	0.449	22.70
10	2M-4	0.050	1.752	0.648	12.96	4.157	0.385	7.69	5.993	0.555	11.10
11	2M-5	0.024	1.140	0.422	17.98	3.401	0.315	13.10	3.995	0.370	15.41
12	S2-1	0.072	2.146	0.794	11.02	3.412	1.242	17.25	11.285	1.045	14.52
13	S2-2	0.025	1.956	0.724	28.96	8.736	0.809	32.39	4.794	0.444	17.76
14	S2-3	0.059	2.480	0.917	15.54	11.608	1.075	18.23	7.084	0.656	11.13
15	*A niger*	0.021	1.140	0.422	20.09	3.196	0.296	14.09	4.395	0.407	19.38

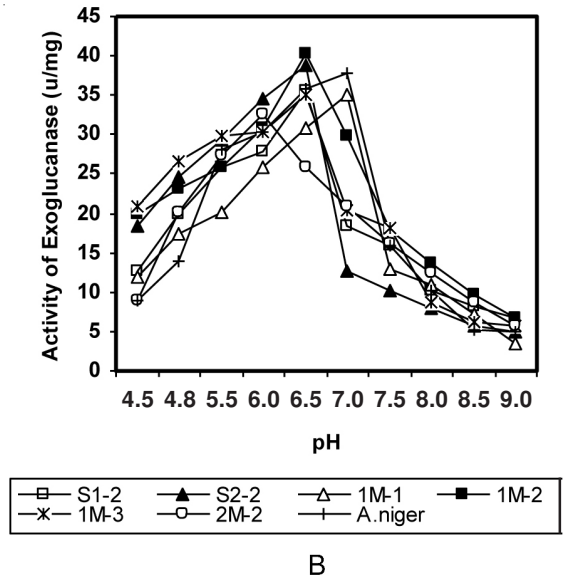


Fig. 2. Temperature (A) and pH (B) optimum. Exoglucanase activity were assayed (A) in acetate buffer pH 4.5 at 37-100 ºC and (B) at optimum temperature and pH various of 4.5-9.0 used acetate buffer (pH 4.5-5.0), phosphate buffer (pH 6.0-7.5) and Tris/HCl buffer (pH 8-9), for 6 isolates that have high activity compared with *A. niger*

Effects of temperature and pH on the activity of endoglucanases: Effects of temperature and pH on the activity of endoglucanases, showed that enzymes from thermophilic bacteria endoglucanases is thermo-tolerant, because at the temperature of 50-100 ºC still show a fairly stable activity in the isolates. On the other hand, sample no. 1M-1, 1M-2 and 1M-3, show an increase in activity up to 100 ºC (Fig. 3 A). Endoglucanases are tolerant of pH ranging from pH 4.8 to 7.5 for consecutive isolates 1M-1, 1M-2 and 1M-3 and tolerant at pH, 4.8 to 7.0 for the isolates, 2M-3, 2M-4 and S2-2, respcctively (Fig. 3B). The optimum conditions of 6 isolates is as follows: isolate 1M-1, 1M-2 and 1M-3 having a maximum activity at 100 ºC and pH 7.5, namely 62.30, 60.50 and 70.50 units/mg, respectively. For 2M-2 and 2M-3 isolates, the maximum activity are 58.85 and 40.32 units/mg at 70 ºC and pH 7. S2-2 isolates reach a maximum activity, *i.e.*, 58.25 units/mg at 80 ºC and pH 7.0. Furthermore, the highest activity of endoglucanase produced by isolates 1M-3 is 70.50 units/mg. compared with the pure enzyme from *Aspergillus niger* with maximum activity of 48.20 units/mg at 40 ºC and pH 7.

Endoglucanases from *Bacillus pumilus* EB3[14], was reported to have the highest maximum activity of 0.079 units/mL at optimum temperature of 60 ºC and pH 6.0. *Bacillus licheniformis* WBS1 and *Bacillus* sp WBS3[15] have maximum activity of 0.118 IU/mL (optimum temperature 60 ºC and pH 8.0) and 0,088 IU/mL (optimum temperature 60 ºC and pH 9.0), respectively. The optimum temperature and pH for rEglA from A niger VTCC-F021 in *Pichia pastoris* activity were 55 ºC (22.39 U/mL) and pH 6.5[16].

Effects of temperature and pH on β-glucosidases activity: Effects of temperature and pH on the activity of β-glucosidase, from 6 isolates of thermophilic bacteria have a higher activity than the cellulase from *Aspergillus niger*, respectively, S1-2, 1M-1, 1M-2, 1M-3, 2M-2 and 2M-3. Optimum

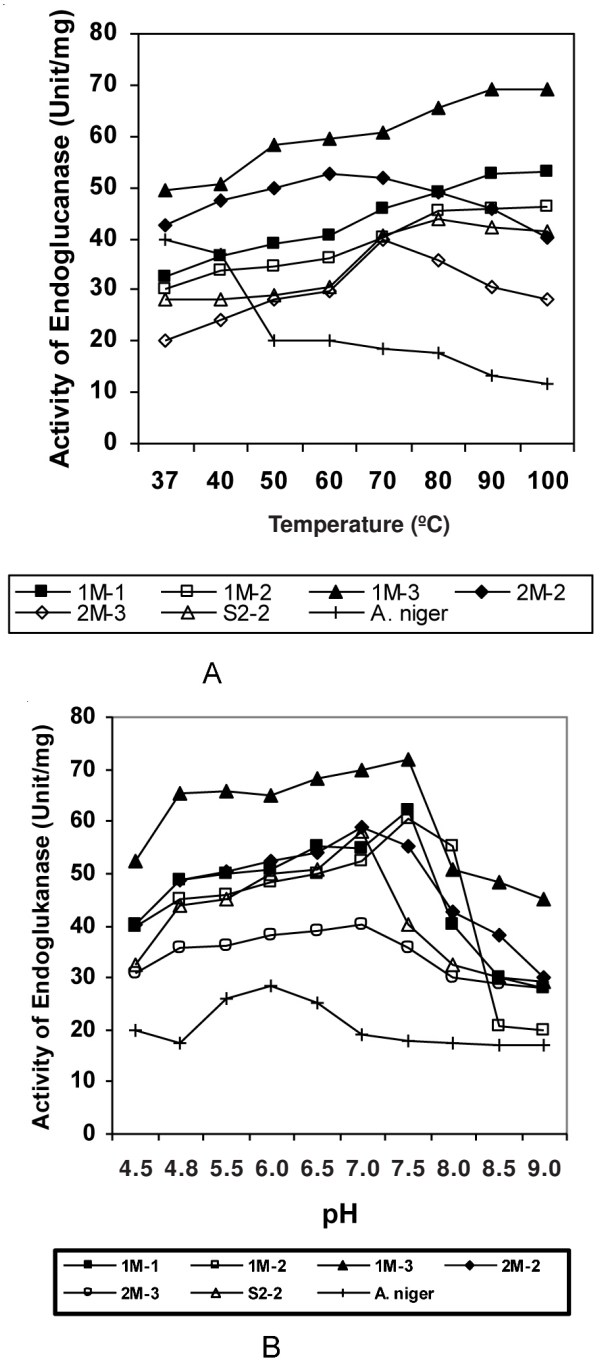


Fig. 3. Temperature (A) and pH (B) optimum. Endoglucanase activity were assayed (A) in citrate buffer pH 4.8 at 37-100 ºC and (B) at optimum temperature and pH various of 4,5 -9, used citrate buffer (pH 4.5-5.0), phosphate buffer (pH 6.0-7.5) and *Tris*/HCl buffer (pH 8-9), for 6 isolates that have high activity compared with *A. niger*

conditions of the isolates S1-2 and 1M-1 temperature of 80 ºC and pH 7 with maximum activity respectively 58.06 and 55.83 units/mg. Condition optimum for 1M-2 isolates and 1M-3 at 80 ºC and pH 7,5 with maximum activity 61.8 and 65.3 units/mg, respectively. 2M-2 isolates and 2M-3, the optimum temperature of 70 ºC and pH 6.5 with maximum activity 62.60 and 45.50 units/mg (Fig. 4A-B). The highest maximum activity of β-glucosidase produced by the, 1M-3 isolates is 65.30 units/mg at optimum conditions. This results higher than the pure enzyme from *Aspergillus niger* with maximum activity of 48.20 units/mg at 37 ºC and pH 7. The maximum activity

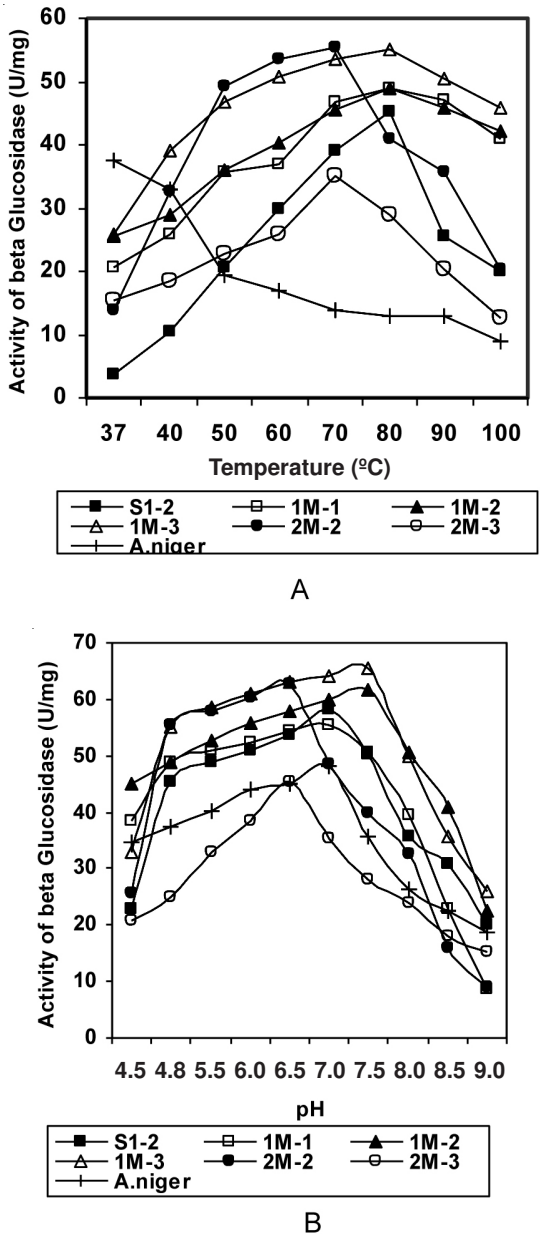


Fig. 4. Temperature (A) and pH (B) optimum. β-Glucosidase activity were assayed (A) in citrate buffer pH 4.5, at 37-100 ºC and (B) at optimum temperature and pH various of 4,5 -9 used citrate buffer (pH 4.5-5.0), phosphate buffer (pH 6.0-7.0) and Tris/HCl buffer (pH 7.5-8), for 6 isolates that have high activity compared with *A. niger*

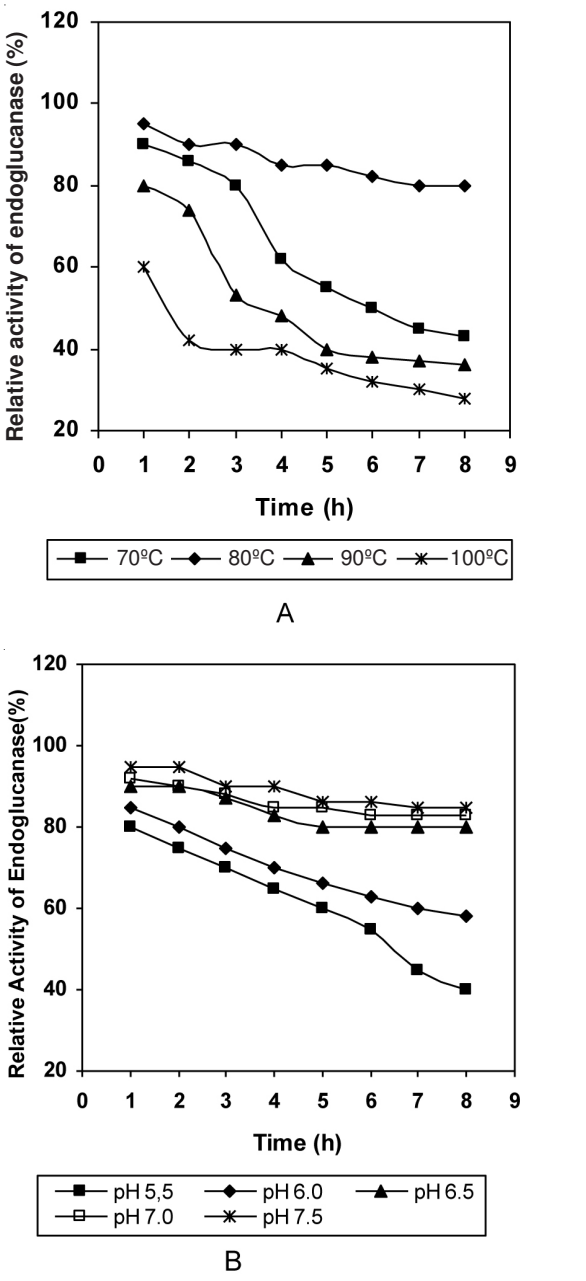


Fig. 5. Temperature (A) and pH (B) stability for 1M-1 isolate. Endoglucanase activity was assayed (A). The enzymes was incubated in citrate buffer pH 4.8 at 70-100 ºC for 1-8 h. Each activity treated at 70, 80, 90, 100 ºC for 0.5 h was taken as 100 % and (B) at pH various of 4.5-9 used citrate buffer (pH 4.5-5.5), phosphate buffer (pH 6.0-7.0) at 80 ºC and incubation time 0.5 h was taken as 100 %

of endoglucanase, higher than exoglucanase and β-glucosidase at optimum conditions of each enzyme. Reported β-glucosidase from *Bacillus pumilus* EB3[15], the highest maximum activity of 0.038 units/mL at 40 ºC and pH 4.8.

Temperature and pH stability: Thermal stability of the enzyme endoglucanase produced by 1M-1 isolates were incubated for 1-8 h at temperatures varying from 70 to 100 ºC is obtained, thermal stability of the enzyme at 80 ºC and was still be able to retain 80 % of its activity after incubation up to 8 h (Fig. 5A) and in pH range of 6.0-7.0 (Fig. 5B), 1M-2 isolates can be retain 90 % of its activity in the treatment incubation temperature of 70 ºC in 6 h and 85 % at 8 h (Fig. 6A) and in pH range of 7.0-7.5 (Fig. 6B). 1M-3 isolates was still be able to retain 82 % of its activity in the treatment incubation temperature of 80 ºC in 8 h (Fig. 7A) and in pH range 7.0-7.5 (Fig. 7B).

Conclusion

Thermophilic bacteria isolated from hot springs Rambo Panti obtained 46 colonies and 14 isolates had cellulase activity (cellulolytic), three enzymes that act on the cellulolytic system, endoglucanase has the highest activity compared to, β-glucosidase and exoglucanase. Six isolates had high activity compared with pure cellulase enzyme from *Aspergillus niger*, for the 6 isolates assayed the effect of temperature and pH showed, endoglucanase tolerant of temperature and pH and shows broad stabilities of temperature and pH, is expected to have potential application in the industry.

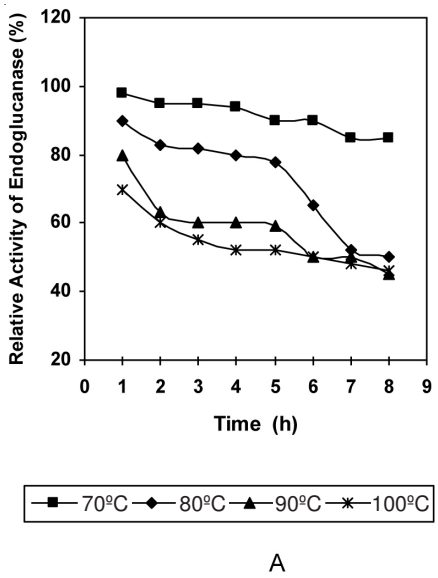
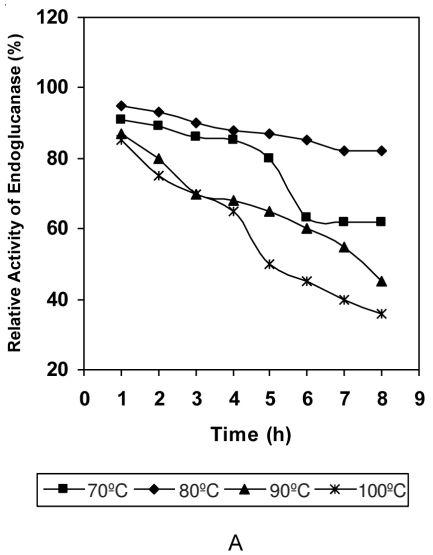


Fig. 6. Temperature (A) and pH (B) stability for 1M-2 isolate. Endoglucanase activity was assayed (A). The enzymes was incubated in citrate buffer pH 4.8, at 70-100 ºC for 1-8 h. Each activity treated at 70, 80, 90, 100 ºC for 0.5 h was taken as 100 % and (B) at pH various of 4.5 -9 used citrate buffer (pH 4.5-5.5), phosphate buffer (pH 6.0-7.0) at 80 ºC and incubation time 0.5 h was taken as 100 %



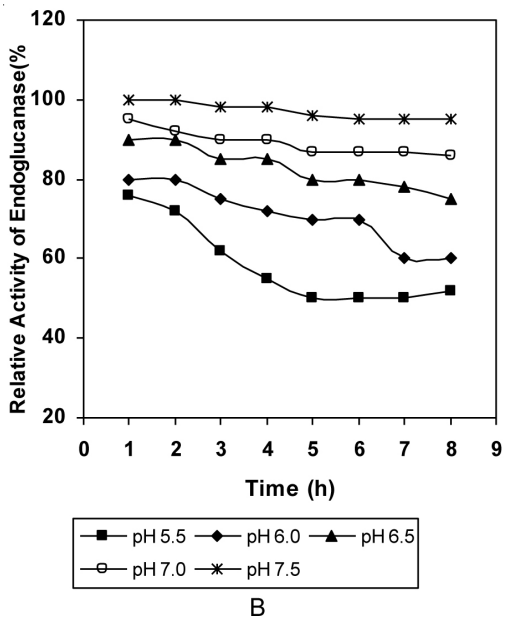


Fig. 7. Temperature (A) and pH (B) stability for 1M-3 isolate. Endoglucanase activity was assayed (A). The enzymes was incubated in citrate buffer pH 4.8, at 70-100 ºC for 1-8 h. Each activity treated at 70, 80, 90, 100 ºC for 0.5 h was taken as 100 % and (B) at pH various of 4.5 -9 used citrate buffer (pH 4.5-5.5), phosphate buffer (pH 6.0-7.0) at 80 ºC and incubation time 0.5 h was taken as 100 %

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