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Effect of pH and Temperature on *Bacillus* sp. R2 Chitinase Activity and Stability

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Abstract

The purified chitinase of Bacillus sp. R2 showed a molecular weight of 41.68 KDa, an optimal pH and temperature of 7.5 and 40°C respectively. The enzyme was stable between pH 7 to 8 but it lost 57% and 62% of its initial activity at the pH 6 and 9 respectively. The purified chitinase also exhibited complete heat stability for 1 h at 40°C and retained above 50% and 30% of its original activity after 30 min heating at 50°C and 60°C respectively. The chitinase activation energy for chitin hydrolysis was 12.93 Kcal/mol. To the best of our knowledge, this is the first study about marine chitinase characterization from novel gram variable Bacillus isolated from the Red sea.
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Keywords: Bacillus sp. R2; chitinase; characterization; pH; temperature; activity; stability.

1. Introduction

Chitinases (EC 3.2.1.14) that catalyze chitin hydrolysis have gained intensive research interest for their versatile biotechnological applications that ranging from chitinous wastes management and phytopathogens biocontrol [1,2]

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to, diagnostics, therapeutic nutracetical s [3,4] and as efficient biomarker in cancer disease [5,6]. Microbial chitinases particularly have received a growing interest over the last decade due to their diverse properties and technological characteristics and were produced and purified from many microbial sources including bacteria, actinomycets, yeasts, fungi , and protozoan species [7, 8, 9, 10] but Bacteria still the best source and their chitinases were most suitable, several bacterial chitinases have been purified and characterized and their investigated biochemical properties are very diverse, which enable them for useful commercial processes. Strong efforts and experiment worldwide are conducted to find new efficient chitinases with novel characteristics that meet to the increased industrial demands. In previous papers [11, 12] Bacillus sp.R2 chitinase was produced and purified, whereas the present work is also a successful effort in this direction which has been carried out with the aim to characterize a marine chitinase from *Bacillus* sp. R2 via determining its molecular weight, its optimum pH and temperature for activity and stability, moreover calculates the chitinase activation energy (Ea) for chitin hydrolysis.

2. Materials and methods

2.1. Chemicals

Peptone and yeast extract were obtained from (Oxöid Hampshire, England).While trypton and agar from (Difco Michigan, USA).Beef extract from (BBL-Maryland,USA), crab shell chitin flakes (Win-lab,UK).2 Hydroxy 3,5 dinitrosalselic acid (DNSA) obtained from (Merck, Darmstadt- Germany); Crab shells chitosan, Acryl amide, N.N-Methylene-acry bis amid. N-acetyl glucosamine and bovine serum albumin (BSA) were from (Sigma -USA). All other chemicals used in this study are of reagent grade and were obtained from commercial sources.

2.2. Bacterial strain and chitinase production and purification

*Bacillus sp.*R2(strain accession number in NCBI GenBank was: DQ923161) was newly isolated marine bacterial strain from red sea, Egypt and identified biochemicaly and molecularly by Cheba et al 2006 .The chitinase enzyme produced from Bacillus sp. R2 in shake flasks (submerged fermentation) using shell colloidal chitin as essential carbon source. The enzyme was purified to homogeneity using affinity chromatography on swollen crab shell chitin column as reported in details in the paper of Cheba et al. 2015 [12].

2.3. Chitinase assay and protein content determination

For chitinase assay, colloidal chitin was selected as substrate and chitinase activity was analyzed according to the method of Miller (1959) [13] by estimating the released reducing sugars spectrophotometrically at 540 nm. A standard curve was established prepared with a series of dilutions of N-acetyl – D-glucosamine (NAG) and DNSA .One unit of chitinase activity was defined as the amount of enzyme required to release 1 μ mol of NAG per minute during reaction conditions.The amounts of protein in crude as well as purified enzyme were determined by the methods of Bradford (1976) [14] and Lowry et al. (1951) [15] respectively, using bovine serum albumin as standard.

2.4. Chitinase characterization

2.4.1. Molecular weight determination

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10%) was performed as described earlier by Laemmli (1970) [16] (Fermentas kit) was used as a protein molecular weight marker. On the other hand, the molecular weight of the enzyme was also calculated arithmetically on the basis of semi logarithmic plot of the bands mobilities on SDS - PAGE using a standard curve established with proteins of known molecular weight.

2.4.2. Effect of pH on chitinase activity and stability

The optimum pH for chitinase activity was determined by incubating the purified enzyme at 37ºC for 30 min at different pH values (5 – 11) using squid chitin as a substrate. The following buffers were used: 25 mM sodium acetate (pH 5 and 6), 25 mM Tris-HCl (pH 7, 7.5, 8 and 8.5) and 25 mM carbonate buffer for (pH 9, 10 and 11). The

enzyme pH stability was determined by preincubating the enzyme solution at various pHs without substrate for 10 min, and immediately cooled down to 0ºC. Relative activity (RA) was calculated according to the following formula (Gunaratna 1993) [17] using the standard assay as the control.

$$\text{Relative activity (RA)} = \frac{\text{EA in control - EA in reated}}{\text{EA in control}} \times 100$$

where: EA is enzyme activity.

2.4.3. Effect of temperature on chitinase activity and stability

The optimum temperature for chitinase activity was determined by incubating the reaction mixture of enzyme substrate for 30 min at different temperatures (10 – 70ºC) at pH 7.5. Chitinase thermostability was examined by preincubating the enzyme solution without substrate for different time intervals (5, 15, 30, 45 and 60 min) at various temperatures (40, 50, 60 and 70ºC). After termination of the reaction by cooling at 0ºC, the substrate was added, the assay was conducted and the relative activity was calculated as described earlier.

2.4.4. Determination of chitinase activation energy

The chitinase activation energy (Ea) for chitin hydrolysis was determined experimentally by measuring the reaction rate constant (K) or (the maximal velocity) at different temperatures (10 to 70ºC) and calculating the log K from Arrhenius equation (Segel, 1993) [18] .

$$\log_{10} \text{K} = -\frac{\text{Ea}}{2.303\,R} \times \frac{1}{\text{T}} + log_{10}\,A$$

where:

K: specific or observed reaction rate constant;
Ea: activation energy (Kcal. $mole^{-1}$) or ($J.mol^{-1}$);
R: Gas constant (1.987 cal / mol / ºK);
T: absolute temperature (ºK);
A: enzyme activity.

After plotting $\log_{10}$ K or $\log_{10}$ V_{max} versus 1/T (Arrhenius plot), the activation energy was estimated from the slope of the linear portion of the plot.

$$\text{Slope} = -\text{Ea} / 2.303 \cdot \text{R} \cdot \text{T} = \frac{\log_{10} \text{K}_2 - \log_{10} \text{K}_1}{(1/T_2 - 1/T_1) \cdot 10^{-3}}$$

3. Results and discussion.

3.1. Determination of chitinase molecular weight

The purified chitinase from Sephadex G-100 was loaded onto 10% SDS-PAGE to determine the molecular weight. Fig. 1 displays the migration of the enzyme as a single band with approximately molecular weight of 41.68 KDa suggesting, that the purified chitinase was homogeneous with one subunit (monomer). On the other hand, the molecular weight of the enzyme was also calculated arithmetically on the basis of semi logarithmic plot of the bands mobilities on SDS - PAGE using a standard curve established with proteins of known molecular weight Table 1). The chitinase molecular weight was found to be 41.68 KDa. Similar molecular weights were found in many marine and terrestrial chitinase for example; 45 KDa for chitinase C of *Alteromonas* sp. [19] 47.7 KDa for *Microbulbifer degradans* [20] and 41 KDa for chitinases from *Bacillus* sp. BG-11[21], *Bacillus* sp. [22] and *B. subtillus* TMR-NK1 [23], respectively.

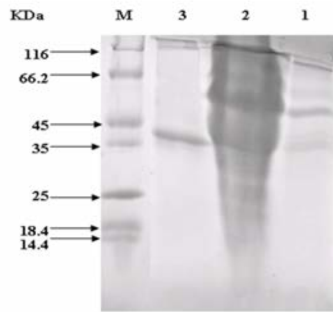

Fig. 1. SDS-PAGE (10%) gel electrophoresis of Bacillus sp.R2 samples. Lane1: R2 crude chitinase, lane2: 80% (NH4)2SO4 concentrated crude enzyme, lane 3: purified chitinase after two successive chromatographic columns (affinity then Sephadex G-100). Lane M: molecular weight protein marker.

Table 1. Relation between molecular weight of standard protein markers as well as Bacillus sp. R2 purified chitinase enzyme and their relative mobilities when applied to 10% SDS-PAGE

Protein/ source	Molecular weight (KDa)	Log_{10} Molecular weight	Relative mobilities (Rm)*
B-galactosidase /E.coli	116	2.064	0.0595
Bovine serum albumin/bovine plasma	66.2	1.820	0.142
Ovalbumin/chicken egg white	45	1.653	0.309
Lactate dehydrogenase/porcine muscle	35	1.544	0.380
Restriction endonucleaseBsp981/E.coli	25	1.397	0.595
B-lactoglobulin/bovine milk	18.4	1.264	0.678
Lysozyme/chicken egg white	14.4	1.158	0.714
purified chitinase	41.68	1.62	0.357

* Rm is the relative mobility of a given protein in the gel when compared to the mobility of the dye.

3.2. Effect of pH on chitinase activity and stability

The pH activity profile of the chitinase was bell shaped, with maximum activity at pH: 7.5 (Fig. 2(a)). The enzyme was still active at pHs7 and 8. Determination of chitinase pH stability indicated that the enzyme was stable between pH 7 to 8 and retained more than 90% of its activity in this range (Fig. 2(b)), but it lost 57% and 62% of its activity at the pHs 6 and 9, respectively. Similar pH stability ranges (7-8) were obtained with chitinase produced from *Bacillus* sp. 13.26[24] and with chitinase of the marine bacterium *Microbulbifer degradans* 240 [25].

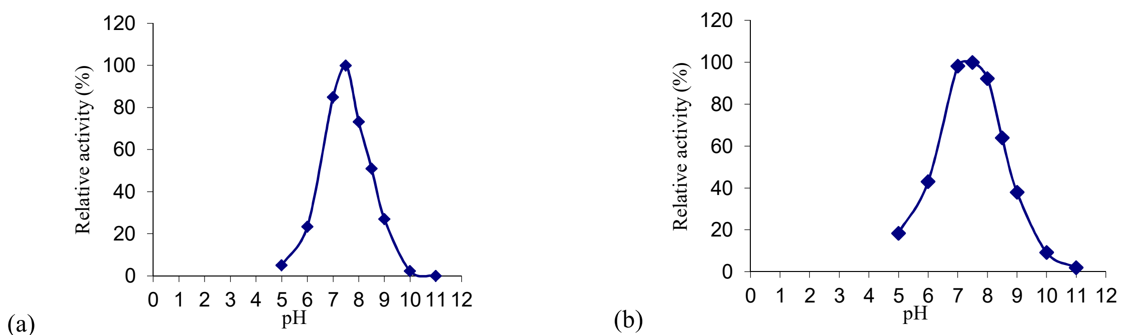

Fig. 2 (a) Effect of pH on chitinase activity; (b) Effect pH on chitinase stability.

3.3. Effect of temperature on chitinase activity and stability

The purified chitinase showed quite good activity over the temperature range of 30 – 45ºC, but the optimum temperature was 40ºC as evident from Fig. 3. Similar optimal temperature was obtained for the chitinase of *Pseudomonas aeroginosa* K187 [26] *Enterobacter agglomerans* [27] and *Streptomyces albovinacieus* [28] ,and 45ºC to 65ºC for the chitinase of the genus Bacillus[21,24,29] The purified chitinase exhibited a complete heat stability for 1hr at 40ºC and retained above 50% and 30% of its original activity after 30min heating at 50ºC and 60ºC, respectively but was instable at 70ºC Fig. 4. These results were in accordance with other reports in literatures such as chitinase of the marine bacterium *Vibrio alginolyticus* 11-8 which was stable at 40ºC for 30 min [30] and the chitinase of *Vibrio* sp. p. 6-1 was also stable at 40ºC but completely inactivated at 50ºC in 30 min [31].

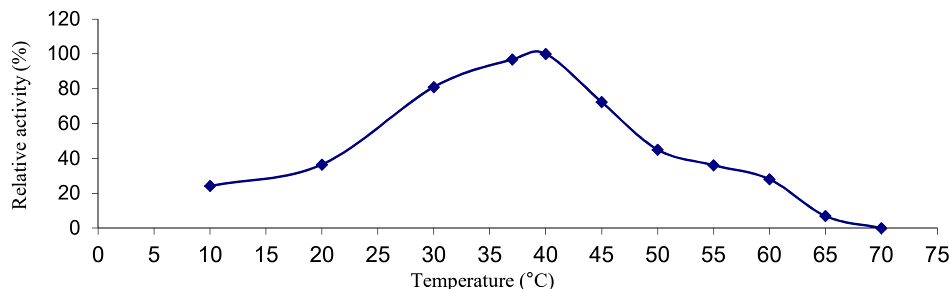

Fig. 3. Effect of temperature on chitinase activity

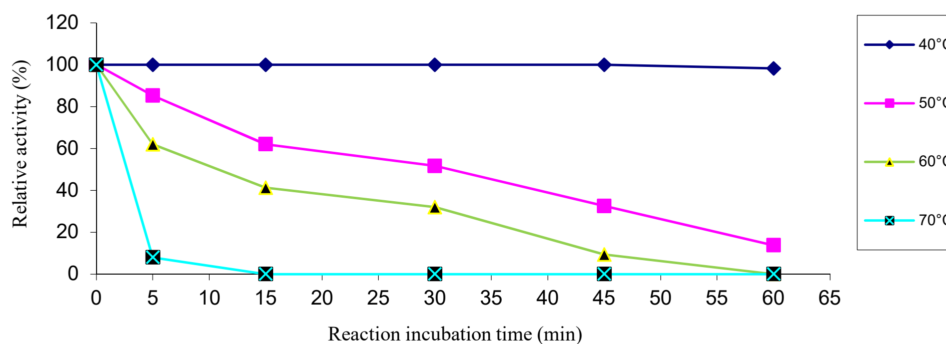

Fig. 4. Effect of temperature on chitinase stability: (chitinase thermostability).

3.4. Determination of chitinase activation energy

It was well known that the minimum energy required for a fruitful reaction was called energy of activation (Ea) [22]. The chitinase activation energy for chitin hydrolysis was investigated throughout the effect of temperature on the initial velocity over the range of 10 to 70ºC, and was estimated from Arrhenius plot as shown in (Fig. 5). A sudden drop in the plot at 45ºC indicated enzyme inactivation above this temperature. The activation energy calculated from the slop of linear portion (below 45ºC) of the plot was 54.15 J/mol or 12.93 Kcal/mol. This value was higher than that reported for *B. subtilis* chitosanase 0,96 Kcal/ mol[23] and *Streptomyces* sp. N06 chitosanase 10.4 Kcal/mol[32] but lower than *Penicillium islandicum* chitosanase (20 Kcal/ mol) [33].

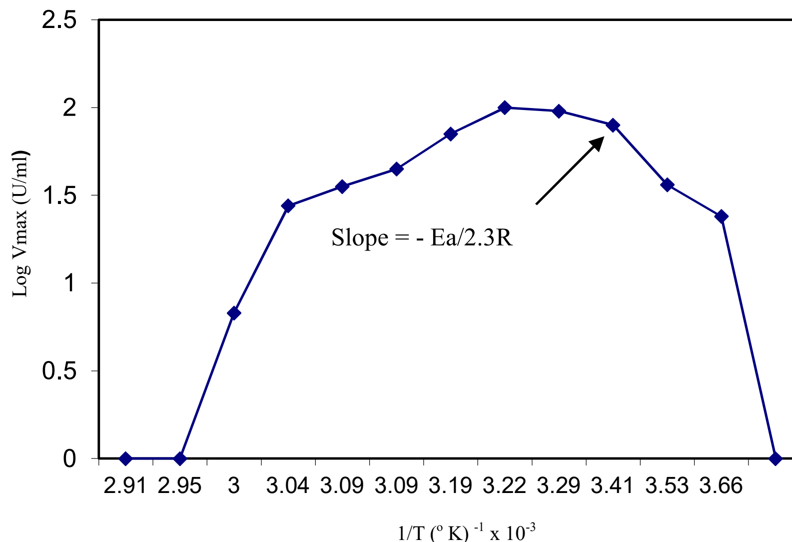

Fig. 5. Arrhenius plot of log V versus 1/T (o K) -1 x 10-3 with slope = Ea/2.3R to calculate the activation energy of chitin hydrolysis by purified chitinase

4. Conclusion

The conclusion drawn from the present study is that *Bacillus* sp. R_2 chitinase showed a molecular weight of 41.68 KDa and 12.93 Kcal/mol which is the first reported activation energy for chitin hydrolysis by marine chitinase from novel gram variable Bacillus isolated from the Red sea. In contrast the enzyme exhibits a weak thermo stability which is the major advantages and the most important challenges for industrial catalysis and need more improvements for future exploitations. Furthermore its clear that the concerted understanding of various aspects and properties of bacterial chitinases will be necessary for promoting chitinase research toward biotechnological applications.

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