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Kinetics Properties of Marine Chitinase from Novel Red Sea Strain of Bacillus

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Abstract

An aerobic Gram variable rod shaped polarly flagellated marine bacterium abbreviated R2 attracted our attention not by its hyper chitinolytic activity but also by its multiple enzymes production (protease, gelatinase, lipase, amylase, dextranase,alginase, arabinase, agarase, etc.). This bacterial isolate was selected and identified using conventional methods as well as 16S rRNA technique and submitted in the Gen Bank sequence database as Bacillus sp. R2 with a given accession number DQ 923161. Its purified chitinase showed a molecular weight of 41.68 KDa. Kinetics study revealed that the chitinase exhibited Km, Vmax and Kcat values of 6.971 mg/ml, 69.63 U/ml and 1.815 µcatal for colloidal chitin, 3.334 mg/ml, 83.32 U/ml and 2.172 µcatal for squid chitin respectively. Furthermore the enzyme catalytic efficiency ($Kcat/K_m$) was estimated to be 0.260 and 0.651 µM $P.min^{-1}mol^{-1}mg^{-1}S$ toward colloidal chitin and squid chitin, respectively. This is the first contribution about marine chitinase kinetics study from novel gram variable Bacillus isolated from the Red sea.

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Keywords: Bacillus sp. R2; 16S rRNA identification; chitinase; purification; kinetics.

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1. Introduction

Chitin, a β- (1-4) homopolymer of N-acetyl - D- glucosamine (GlcNAc), is the second most abundant polysaccharide existing in nature after cellulose. Chitinases (E.C.3.2.1.14), which are distributed in variety of organisms in the biological world, are a glycosyl hydrolases that act on the β-(1,4) linkage of chitin polymer. [1] During the last decade, chitinases have received an increased attention due to their wider ranges of biotechnological applications especially in the biocontrol of fungal phytopathogens [2] and harmful insects [3]. They have also been used as vaccine [4] as well as in the preparation of pharmaceutically important chitooligosaccharides [5,6].

It was well known that the substrate type and concentration affect the chitinase activity and subsequently the k_m and Vmax values. The numerical value of the Michaelis constant (K_m) is of interest for some reasons, among then knowing the Km value can help to adjust the assay condition so that the [S] > Km and there by determine the V-max moreover give an idea about the apparent affinity for the enzyme to its substrate. (The best substrate is that which has the highest Vmax/Km ratio). A wide range of bacterial chitinases particularly were, purified, and characterized7, 8, 9, 10] however very little reports or papers described their kinetics, for this reason the present work was a detailed study which has been carried out with the aim to determine all the kinetics parameters of Bacillus sp. R2 chitinase which was previously produced, purified, and characterized [11,12,13,14].

2. Materials and methods

2.1. Substrates and chemicals

Chitin was extracted from crustaceans and squid by the method of "Synowiecki*et al*. 1982 [15]", Crab shell chitin flakes (Win-lab, UK).Swollen chitin was prepared according to "Monreal and Reese, (1969) [16]"Peptone ,tryptone, and yeast extract were obtained from (Oxöid Hampshire, England). N-acetyl glucosamine, and bovine serum albumin (BSA) were from (Sigma -USA), 2 Hydroxy 3,5 dinitrosalselic acid (DNSA) obtained from (Merck, Darmstadt- Germany). All other chemicals and reagents that were used were of highest grade commercially available.

2.2. Microorganism and purified chitinase assay

*Bacillus sp.*R2marine bacterial strain isolated and identified biochemically and molecularly by cheba et al 2006 (strain accession number in NCBI GenBank was: DQ923161). The chitinase enzyme was produced , purified to homogeneity[11,12] and characterizedas reported in[13,14]. Chitinase activity was analyzed according to the method of "Miller (1959) [17]" by estimating the released reducing sugars spectrophotometrically at 540nm. 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.

2.2.1. Effect of enzyme concentration on chitinase activity

Different volumes (25, 50, 75, 100 and 200 µl) of the purified chitinase were used in excess of substrate to determine the suitable amount of enzyme to be added to the reaction mixture. Reactions were conducted at pH 7.5 and 37 ◦C for 30 min then the enzyme activity was determined as before.

2.2.2. Effect of reaction incubation time on chitinase activity

Reaction mixtures were incubated at 37°C and pH: 7.5 for various time intervals (0.2, 10, 20, 30, 40 and 60 min) then the enzyme assay were completed and the activity was measured as above.

2.2.3. Effect of substrate concentration on chitinase activity

The influence of substrate concentration on chitinase activity was determined at different concentration of shrimp shell colloidal chitin and squid bone chitin as substrates. The tested concentrations were (0.5, 1, 2, 4, 6, 8, 10, 14 and 20 mg/ml) (w/v).

2.2.4. Kinetics parameters determination

Michaelis - Menten constant (Km) and maximal velocity (Vmax) were determined by nonlinear regression from Michaelis - Menten equation and its derivatives Hanes - woolf, Eadie-Hofstee and Lineweaver-Burk plots using Hyper 32 program-copyright 2003, J.S. Easterby[(K)], version 1.0.0 Hyperbolic regression analysis of enzyme Kinetic data (available from: http://homepage.ntlworld.com/John.easteby).The catalytic constant (K_{cat}) or turn over number (T.O.N) of the enzyme toward colloidal chitin and squid chitin substrates was estimated according to the following equation[18]:

$$K_{cat} = \frac{Micro\ equivalent\ of\ substrate\ transformed.\ min^{-1}}{Micro\ equivalentweight\ of\ enzyme} \quad (1)$$

3. Results and discussion

3.1. Effect of enzyme concentration and reaction incubation time

The chitinase activity showed enzyme concentration relatedness as evident in the Fig. 1(a) the range 50-100µl of the enzyme was the suitable concentration. The Fig. 1(b) indicated that as the incubation time was increased the chitinase activity increased further incubation more than 40 min did not show a linear relationship. El Sayed et al. found 120 min incubation at 40ºC gives the maximal activity for the chitinase of *Streptomyces albovinaceus* and more than 120 min adverse effect was obtained [19] may be due to protein denaturation[20].

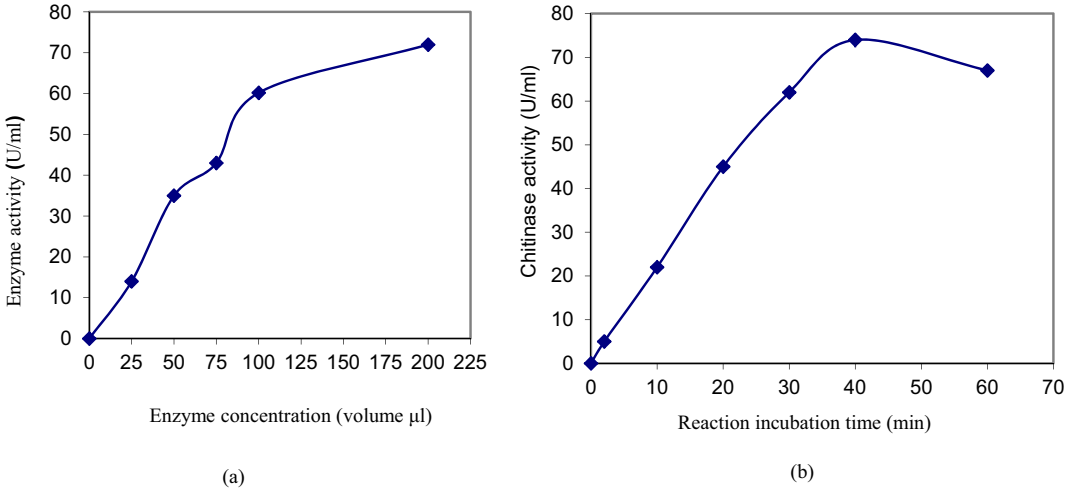


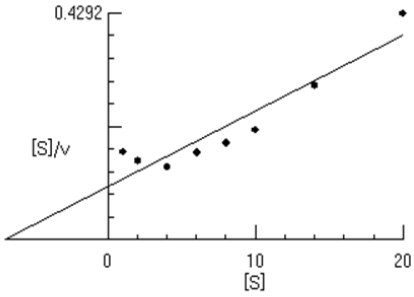
Fig. 1. (a) Effect of enzyme concentration on chitinase activity; (b) Effect of reaction incubation time on chitinase activity

3.2. Effect of substrate concentration and Kinetics parameters determination

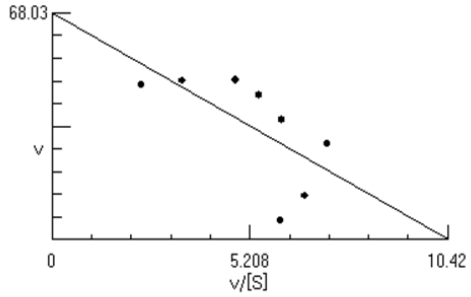
As cited in the materials and methods, the kinetic parameters were calculated from the nonlinear regression using Michaelis-Menten equation and its derivatives: Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee plots. The

results presented in the Figs. 2,3 and summarized in the Table (1) revealed that *Bacillus sp.* R_2chitinase exhibited a high affinity toward squid chitin than colloidal chitin with Km of 3.334 and 6.971 mg/ml respectively, V-max of 83.32 and 69.63 U/ml respectively and with V-max/Km of 24.991 and 9.988 mg/ml respectively, these data confirmed the results of the substrate specificity which showed that the enzyme prefer the β chitins of squid and calmar than the α chitins of shrimp and crabs shells. On the other hand nearly similar result were obtained by many workers for example the chitinase of *B.cereus* 28-9 possessed a Km of 4.1 mg/ml for colloidal chitin azure[21] and chitinase of *Sanguibacter sp.* c4 have a Km of 6.95 mg/ml for colloidal chitin[22] where as *Enterobacter sp.*NRG4 chitinase exhibited a V-max of 83.33, 74.07, 40 and 33.33 µmole/µg/h for swollen chitin, colloidal chitin azure, regenerated chitin and glycol chitin respectively. Furthermore the chitinase of *Bacillus sp.* BG11 exhibited a Km value of 12mg/ml against swollen chitin[23] and the four chitinases of *Aeromonas schubertii*[24] exhibited the Kms 2.9, 2.6, 2.6, 5.5mM toward colloidal chitin and the Vmaxs 0.87, 1.92, 0.72 and 1.10 U/mg respectively, where as the two chitinases of *Vibrio alginolyticus*H-8[25] has the Kms of 1.4 and 0.8 mg/ ml against squid chitin[25] and *Mucor mucedo* has the Km and Vmax of 16.7mg/ml and 2.33 mM/min/mg toward chitin[26].

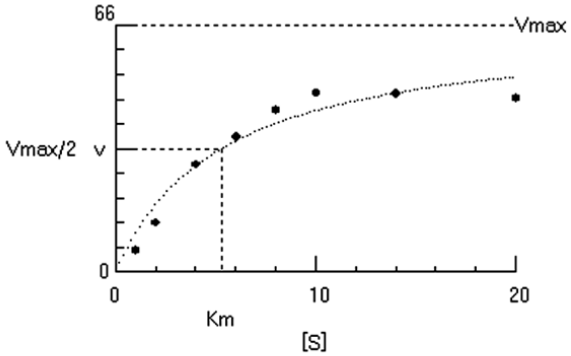
The catalytic constant and the catalytic efficiency (Kcat/Km) of *Bacillus sp.* R_2chitinase were also calculated and the results registered in the Table (1) indicated that the enzyme possessed Kcat and Kcat/Km of 1.815 µ catal and 0.260 µM $Pmin^{-1}$ mol^{-1} mg^{-1}S for colloidal chitin and 2.172 µ catal and 0.651 µM $Pmin^{-1}$ mol^{-1} mg^{-1}S for squid chitin.



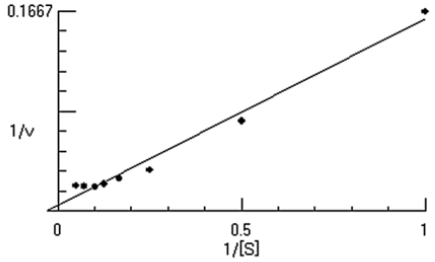
(a) Hanes-Woolf



(b) Eadie-Hofstee

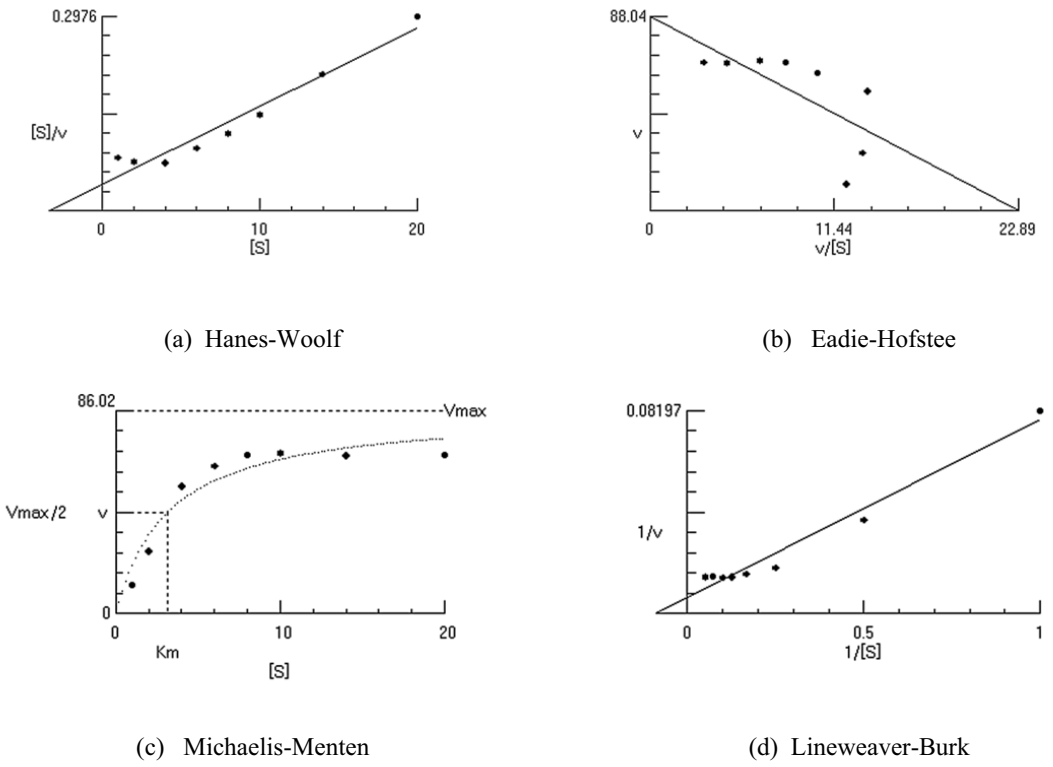


(c) Michaelis-Menten



(d) Lineweaver-Burk

Fig. 2. *Bacillus sp.* R_2chitinase Kinetics plots (a, b, c ,d) against colloidal chitin



(a) Hanes-Woolf (b) Eadie-Hofstee

(c) Michaelis-Menten (d) Lineweaver-Burk

Fig. 3. *Bacillus sp.* R_2chitinase Kinetics plots (a, b c, d) against squid chitin

Table 1. Kinetic parameters of Bacillus sp. R2chitinase against colloidal chitin and squid chitin substrates

plots	Km (mg/ml)	Vmax (U/ml)	Vmax/Km (U/mg)	K_{cat} (µcatal)	K_{cat}/Km(µMP min^{-1} mol^{-1} mg^{-1} S)
	S1- S2	S1- S2	S1- S2	S1- S2	S1- S2
Hanes-Woolf	6.97- 3.33	69.63- 83.32	9.988 - 24.99	1.81- 2.17	0.26 - 0.65
Eadie-Hofstee	6.53- 3.84	68.03- 88.04	10.41- 22.89	1.77- 2.29	0.27 - 0.59
Michaelis-Menten	5.82- 3.53	66.00 - 86.02	11.34- 24.35	1.72- 2.24	0.29 - 0.63
Lineweaver-Burk	5,88- 2.22	68.96- 90.90	11.72- 40.94	1.79- 2.37	0.30 - 1.06

P: product (N-acetyl glucosamine), S: substrate (colloidal chitin or squid chitin),S1: colloidal chitin , S2: squid chitin

Huang and Clen, (2005)[27] found that the tow chitinases chi CH and chi W of *Bacillus cereus* 28-9 exhibited a Kcat of 4×10^{-4}, 1.1/h for colloidal chitin azure and 2×10^{-4}/S, 3.6/h respectively for glycol chitin and showed a Kcat/Km of 2.3×10^{-5}, 2.6×10^{-2} h/mg/ml for colloidal chitin azure and 1.3×10^{-4} S/mg/ml, 0.9 h/mg/ml for glycol chitin. And very recently Suginta, 2007[28] found that the chitinase chi 65 of the marine bacterium *Vibrio alginolyticus 283* displayed 2,3 folds greater catalytic efficiency (Kcat/Km) toward PNP-di NAG than the chitinase chi-90.

4. Conclusion

The main conclusion obtained from the kinetic study of *Bacillus* sp. R_2chitinase that the enzyme exhibited a high affinity toward squid chitin than colloidal chitin; these data emphasized the fact stating that the enzyme prefer the β

chitins of squid and Calmar than the α chitins of shrimp and crabs shells. Therefore, further kinetic analysis and substrate specificity on soluble and oligomeric substrates investigations are needed. This type of experiments expected to makes the enzyme suitable for the preparation of pharmaceutically important chitooligosaccharides, or useful for other biotechnological applications.

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