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Effect of Metal Ions, Chemical Agents, and Organic Solvent on *Bacillus Sp*.R2 Chitinase Activity

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Abstract

Bacillus sp. R_2purified chitinase was subjected to the effect of metal ions, chemical agents, and organic solvent on its activity ,the results showed that K^+, Mn^{2+}, Na^+, Mg^{2+} and Ca^{2+} stimulated chitinase activity by 2,4,7,15 and 21% respectively whereas Cu^{2+}, Fe^{2+}, Zn^{2+}, Ag^+ and Hg^{2+} inhibited the enzyme. 10mM β-mercaptoethanol and EDTA decreased the activity by 38.7 and 78.4% respectively whereas 1% SDS inactivated the enzyme completely, also 1mM galactose, glucose and N-acetyl glucosamine inactivated the enzyme by about 14.4, 25.2 and 38.7% respectively ethanol, acetone and isopropanol inhibited about 30% of chitinase activity however full or nearly full inhibition was observed with chloroform diethylether and ethylacetate. Furthermore the chitinase shelf life was 30 day at -20C and survived 4 freezing and thawing with lost of about 30% of its original activity. This is the first contribution about marine chitinase characterization study from novel gram variable Bacillus isolated from the Red sea.

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*Keywords:*Bacillus sp. R2; chitinase; characterization; metal ions; chemical agents; organic solvent; activity.

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1. Introduction

Chitinases (E.C. 3.2.1.14) hydrolyze the linear polymer chitin, a polysaccharide of β-(1,4) linked *N*-acetylglucosamine (GlcNAc; 2-acetamino-2-deoxy-β-d-glucose) units ,have gained intensive research interest for their versatile biotechnological applications that ranging from chitinous wastes recycling andphytopathogensbiocontrol[1,2]to the preparation of pharmaceutically important chitooligosaccharides[3,4]. A wide range of microorganisms secrete chitinases for multiple purposes such as nutrition, morphogenesis,defence, antagonism, parasitism and pathogenesis [5–6].

Due to their diverse properties and technological characteristics,bacterialchitinases particularly were produced ,purified ,and characterized from many Gram negative and positive genera including, *Serratia,Aeromonas ,Pseudomonas, Alteromonas,Enterobacter, Bacillus ,Clostridium,Micronomospora and actinomycets*[7, 8, 9, 10,11], however no reports or papers were found about marinechitinases from Gram variable Bacilli, for this reason in previous papers[12, 13] we describedchitinase production and purification from marine Gram variable Bacillus sp.R2, whereas the present work was a successful contribution to characterize the enzyme via investigating the effect of certain factors such as metal ions, chemical agents, and organic solvent on chitinase activity.

2. Materials and methods

2.1. Chemicals

Yeast extract and peptone 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, galactose,glucose, N-acetyl glucosamine and bovine serum albumin (BSA) were from (Sigma -USA).All of the other chemicals and reagents that were used were of highest grade commercially available.

2.2. Microorganism and purified chitinase characterization

*Bacillus sp.*R2marine bacterial strain isolated and identified biochemically and molecularly by cheba et al 2006 (strain accession number in NCBI Gen Bank was: DQ923161).The chitinase enzyme was produced and purified to homogeneity as reported in[12,13],moreover the enzyme was subjected to characterization.

2.2.1. Effect of metal ions on chitinase activity

The enzyme was preincubated separately with 1 mM of various metal ions dissolved in 50mM Tris-HCl buffer, pH 7.5 at 37ºC for 30 min. Enzyme activities without metal ions addition (controls) were measured simultaneously and the residual activity was calculated.

2.2.2. Effect of major sea water cations concentration on chitinase activity

The reaction mixture of enzyme and substrate was preincubated separately for 30 min at 37ºC with different concentration (1, 5, 10, 15, 25, 50 and 100mM) of cations dissolved in 50mM Tris –HCl, pH 7.5. The tested major cations were chloride form of Na^{+}, K^{+}, Ca^{+2} and Mg^{+2}.

2.2.3. Effect of chemical reagent and some monosugars on chitinase activity

The enzyme solution was incubated separately in 50 mMTris – HCl, pH 7.5 containing various chemicals for 10 min at 370C. Enzyme activities without adding chemicals (controls)were measured in parallel.The residual activity was determined by adding the substrate and carrying out the enzyme assay under the optimum condition.

2.2.4. Effect of organic solvents on chitinase activity

The enzyme was preincubated separately for 10 min at 37ºC with 50% of the organic solvents. Reaction mixtures were carried out as above.

2.3. Analytical procedures

2.3.1. Chitinase assay

Chitinase activity was analyzed according to the method of Miller (1959) [14] 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.

2.3.2. Protein content assay

The amounts of protein in crude as well as purified enzyme were determined by the methods of Bradford (1976) [15] and Lowry et al. (1951) [16] respectively, using bovine serum albumin as standard.

3. **Results and discussion**

3.1. Effect of metal ions on chitinase activity

Data given in Table 1 revealed an increase in the chitinase activity 2, 4, 7, 15 and 21% by the addition of 1mM of K+, Mn2+, Na+, Mg2+ and Ca2+, respectively, where as other ions had a drastic effect on the enzyme activity and their inhibitory effect increased in the following order: Cu2+ < Fe2+ < Zn2+ < Ag+ < Hg2+. The chitinase activation by Ca2+, Mg2+, Mn2+, Na+ and K+ was reported by many authors[17,18,19] and the inhibition by Hg2+ , Ag2+ , Fe2+ ,Cu2+ and Zn2+ also was noticed in many literatures[17,18,19,20] However, certain ions such Cu2+ and Zn2+ could sometimes act as chitinase stimulators [21,22]and sometimes it could be chitinase inhibitors [17,19,23]

Table 1.Effect of metal ions on chitinase activity.

Metal ions (1mM)	Relative activity (%)	Metal ions (1mM)	Relative activity (%)
None	100	MgCl2	115
KCl	102	MnCl2	104
NaCl	107	FeSO4	34.5
HgCl2	0	ZnSO4	25.3
CaCl2	121	MgSO4	98.6
CuCl2	64.7	AgNo3	12.8

3.2. Effect of major sea water cations concentration

The results registered and graphically illustrated in Figure (1) showed that the four major cations contained in sea water (Na+, K+, Mg2+ and Ca2+) activated the enzyme, among them Ca2+ remarkably increased the activity by about 0.6 times at the concentration 10-50 mM followed by Mg2+ and Na+ which enhanced the activity by about 1.3 fold at the concentration of 5-15 mM and 15-50 mM for Mg2+ and Na+, respectively. Similar findings were obtained by Tsujibo et al. (1992)[17]who found that the chitinase of the marine bacterium *Alteromonas* sp. strain 0-7 was stimulated by the four major seawater cations, but he found also that Mg2+ at the concentration of 1-40 mM enhanced the activity by 1.6 fold instead of Ca2+. Other investigators also discussed the role of Ca2+ ion for the hydrolase activity generally and for the chitinases specially. Wright and Smucker (1986)[24]reported that the chitinase activity of the American oyster in full strength sea water was strongly inhibited by Mg2+, slightly

inhibited by Na+, but highly stimulated by Ca2+. Our chitinase also showed higher activity and was more stable in 75% sea water containing various cations such as Na+, K+, Mg2+ and Ca2+ than in artificial sea water or in 50mM Tris - HCl buffer, pH 7.5 (Data not shown). These results collectively emphasized the fact stating that the enzyme produced from marine organisms may have properties different from those produced by terrestrial organisms. Therefore, it is interesting to investigate the chitinase structure and function relationship.

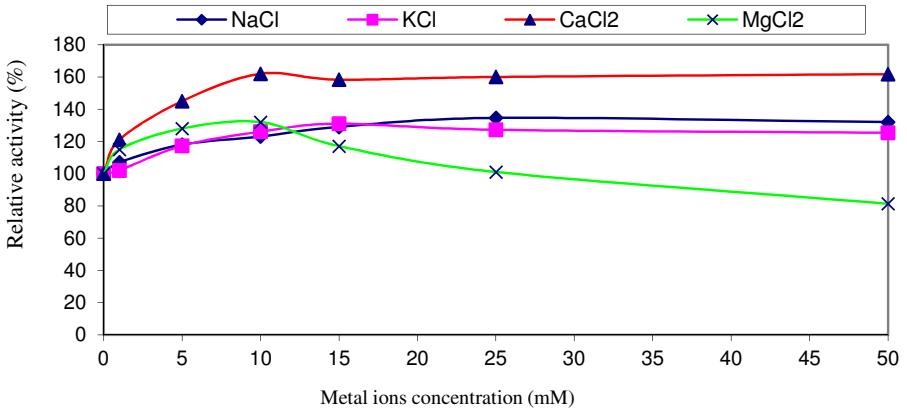

Fig. 1.Effect of sea water major salts concentration on chitinase activity.

3.3. Effect of chemical reagent and some monosugars

The response of chitinase to the action of various chemical modifying reagents was performed and designed with the aim to understand the catalytic mechanism. Table (2) indicated that the chitinase activity was completely eliminated by 1% SDS but 22.5% and 84.3% remained at the concentration of 0.1% of SDS and Triton X-100, respectively. ß -mercaptoethanol and EDTA at a concentration of 1mM inactivated the enzyme by about 26% and 43.1%, respectively but the concentration of 10mM of ß -mercaptoethanol and EDTA decreased the activity by 38.7% and 78.4%, respectively. The other reagents did not have a significant effect on the chitinase. These results were in accordance with those reported in the literature that showed the inhibitory effects of SDS[21,25] Triton X-100[25] β-mercaptoethanol[27 ,28]EDTA [26,28]on the chitinase activity. Table (2) also showed that 1mM of galactose, glucose and N-acetyl glucosamine inactivated the enzyme by about 14.4%, 25.2% and 38.7%, respectively. Sakai et al. (1994) [21]also found that galactose, glucose and N-acetyl glucosamine inhibited the enzyme of B. stearothermophilus by 15%, 30% and 28%, respectively and the chitinases of *Enterobacter* sp. NRG4 [26]and *Metarhizium anisopliae*[29]was inhibited by 19.22 and 81% and 28.79 and 21% after addition 10mM of glucose, D-glucosamine and N-acetyl D- glucosamine, respectively. Others also demonstrated that the chitin hydrolysis end products such as N-acetyl glucosamine, chitobioses and the other chitooligosaccharides inhibited the chitinase activity [30, 21, 28].

3.4. Effect of organic solvents

As shown in Table (3) the chitinase exhibited a moderate tolerance (retained up to 70% of its original activity) to some organic solvents such as ethanol, acetone, isopropanol. However, full or nearly full inhibition by chloroform, diethyl ether, ethylacetate and diethylene dioxide (dioxane) was established. Sakai et al. (1994) [21]also found that butanol, ethanol, propanol, acetonitrile and dioxane decreased the chitinase activity of *Bacillus stearothermophilus* CH-4.

Table 2. Effect of chemical reagents and some mono sugars on chitinase activity

Chemical reagents	Concentration	Relative activity (%)	Chemical reagents	Concentration	Relative activity (%)
None	0 - 0	100 - 100	PMSF	3 mM	96.3
SDS	0.1- 1%	22.5 - 0	Ammonium sulfate	1mM	83.69
Triton X-100	0.1- 1%	84.3 - ND	Tween 20	0.5%	94.2
Urea	0.1- 1%	92.6 - 83.5	Glycerol	0.5%	96.8
β-mercaptoethanol	1-10 mM	74 - 61.3	Glucose	1mM	74.8
EDTA	1-10 mM	56.9 - 21.6	Galactose	1mM	85.6
KCN	1mM	98	N-acetyl glucosamine	1mM	61.8

Table 3. Effect of organic solvents on chitinase activity

Organic solvent (50%)	Relative activity (%)	Organic solvent (50%)	Relative activity (%)
None	100	Acetone	77.9
Methanol	67.5	Chloroform	0
Ethanol	76	Toluene	8.3
Propanol	37.5	Ethylacetate	2
Isopropanol	70.3	Diethylether	0
Butanol	45.6	Diethylene dioxide (dioxane)	6.5

4. Conclusion

The main conclusion obtained from the characterization of marine chitinase from novel gram variable *Bacillus* sp. R_2that the enzyme showed higher activity and stability in 75% sea water containing various cations such as Na+, K+, Mg2+ and Ca2+.These results collectively emphasized the fact stating that the enzyme produced from marine organisms may have properties different from those produced by terrestrial organisms. Therefore, further investigation is needed such as chitinase structure and function relationship site directed mutagenesis and enzyme conditions optimizations which expected to makes the enzyme industrially useful for biotechnological applications

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