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Bacillus Sp. R2 Chitinase: Substrate Specificity, Shelf-Life Stability, and Antifungal Activity

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Abstract

The suitability of different substrates for Bacillus sp. R2 chitinase activity was tested on various crude and purified chitinous substrates, as well as chitinase storage stability was studied weekly for 2 months at three different temperatures (25, 4 and -20°C). The results revealed that chitinase enzyme of *Bacillus sp*. R2 catalyzed the hydrolysis of various chitinous substrates, and exhibited greater activity towards the chitins β of Calmar and squid. The temperature -20ºC was the most appropriate temperature for chitinase storage where the enzyme exhibited a relative resistance to freezing and thawing conditions, where its live 4 cycles (4 weeks) with missing 30% from the initial activity. On the other hand, at the temperatures 4 and 25ºC the enzyme retained 43.6% and 44.9% of its initial activity after the first month and 12% and 27.6 after the second month. Concerning the antifungal activity, the chitinase enzyme displayed a mild effect against many saprophytic and plant pathogenic fungi according the descending order: Aspergillus *niger* > *Penicillium degitatum*> *Aspergillus flavus* > *Fusarium calmorum* > *Penicillium sp.* >*Macrophomira sp.* > *Rhizoctonia solani*. This type of studies characterizes the enzyme for potential pharmaceutically important chitooligosaccharides preparation or promising antifungals production.

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Keywords: Bacillus sp. R2; chitinase; substrate specificity; shelf-life stability; antifungal activity.

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1. Introduction

Chitinases are very important category of glycoside hydrolases (GH) that play a key role in chitin degradation, naturally abundant polysaccharides [1]. Recently, they attracted tremendous attention due to their wider biotechnological applications, particularly in the agricultural field, where can be used as promising biopesticides in the control of fungal, nematode and insect pests [2]. However, chitinases from different sources have diverse characteristics, mode of actions and affinities for different chitinous substrates; this substrates preference differences (crystalline, acetylated, or soluble) among chitinases have been associated with the substrate-binding modules and seem to restrain optimization procedures and complicate standardization techniques for enhanced practical applications [3, 4]. For these reasons substrate specificities studies and explorations were very important for better understand the varied antifungal properties of chitinases, and develop chitinases with enhanced hydrolytic, or transglosylating and antifungal properties.

In precedent published researches, chitinase enzyme from *Bacillus sp. R2* was obtained [5, 6], optimized [7, 8, 9], purified [10] and well characterized in [11, 12, 13]. The present work, designed to complete *Bacillus Sp*. R2 chitinase characterization, via investigating its substrates specificity, shelf-life stability, and antifungal activity.

2. Material and Methods

2.1. Substrates and chemicals

Crustaceous substrates and chitin were prepared and demineralized by the method of (Synowiecki et al. 1982) [14], Swollen chitin was prepared according Monreal and Reese, (1969) [15] . flaked chitin of Crab shell was supplied from Win-lab company, whereas N-acetyl glucosamine, bovine serum albumin, Crab Shell Chitosan, Avicel, CMC, xylan, pectin, starch, dextran blue and agarose were supplied from Sigma company. The other used chemicals were of good quality.

2.2. Effect of substrate specificity on chitinase activity

The purified enzyme preparation[10], was incubated separately at 37ºC for 30 min with 1% of the following substrates: crude powders of demineralized and non-demineralized crab, shrimp, prawn, squilla shells and squid bone; Chitins of crab, shrimp and prawn shells and chitins of squid bone and Calmar pen; Colloidal and swollen chitin of shrimp and crab shells, respectively; crab shell chitosan with 100% and 70% deacetylation, respectively; Avicel, CMC, xylan, pectin, starch, dextran blue and agarose. The released reducing sugars after incubation with the enzyme were measured and the residual activity was determined as mentioned bellow.

2.3. Determination of chitinase shelf-life stability

The chitinase storage stability was studied weekly for 2 months at three different temperatures: room temperature (25°C), 4°C and -20°C. Enzyme preparation100µl was taken weekly from the stored aliquots and tested for chitinase residual activity.

2.4. Chitinase antifungal activity

The antifungal activity of the purified chitinase was carried out against some phytopathogenic fungi belonging to the following genera: *Fusarium calmorum, Rhizoctonia solani, Macrophomira sp. Aspergillus niger, Aspergillus flavus, Penicillium degitatum and Penicillium sp.* These fungi were cultivated for 7 days on potato dextrose broth medium. At the end of incubation period, the fungal mycelia were separated from the broth, washed several times with distilled water and dried. The purified chitinase solution was assayed on 1% of each homogenized air dried fungal mycelium as substrate, the assay was conducted with two replicates and control samples under the standard condition cited earlier.

2.5. Protein content and chitinase assays

Soluble proteins were estimated by Bradford method [16]. Moreover, chitinase assay was assessed by Miller method at 540nm using 3,5-Dinitrosalicylic acid (DNSA) reagent [17].

3. 3. Results and Discussion

3.1. Effect of substrate specificity on chitinase activity

The suitability of different substrates for the chitinase activity was tested on various crude and purified chitinous substrates. Table (1) revealed that the enzyme exhibited a more moderate activity on the crude demineralized chitinous substrate than the non - demineralized one. On the other hand, highest activity was obtained on both colloidal and pure chitins. This was possibly due to the open structure of colloidal chitin and to the availability of larger number of active sites or termini for the enzyme in purified chitins than the crude chitins. From all the tested substrates the chitinase of this microorganism hydrolyzed various chitinous substrates preferentially and exhibited greater activity towards the chitins β of Calmar and squid. This finding was not surprising since Shigemasa et al. (1994) documented it [18] . Those found that the chitinase isolated from Bacillus sp. strain P1-75 degraded β-chitin more efficiently than α –chitin. This may be attributed to the tightly packed antiparallel strands of α –chitin, which renders it not accessible to the action of chitinase enzyme, and this may also explain why most organisms have α rather than β -chitin in exoskeletons or cell walls [19].

Obishi et al. (1996) [20] also demonstrated that the marine bacterium *Vibrio alginolyticus* produced two chitinases showing a maximum activity (100%) towards squid chitin and 5205% and 2705% towards chitosan for the enzyme C1 and C3 , respectively. Lan et al. 2006 [21] also found that chitinase of *Aeromonas hydrophila* exhibited a specificity towards colloidal chitin and chitosan with low degree of acetylation. Furthermore, Osswald et al. 1993 and 1994[22,23] found that the chitinases of Citrus and sweet orange also possessed chitosanase activities. In addition the chitinase displayed very weak activity (1.3% and 8%) against xylan and CMC, respectively but not on pectin, starch or dextran indicating the presence of a different substrate recognition mechanism in the enzyme.

Table 1. *Bacillus sp*. R2 chitinase substrate specificity.

Substrates Type	Substrate (1%)	Relative Activity	Substrate (1%)	Relative Activity
Crude Substrates	Crab Shell Powder	13	Demineralized Crab Shell Powder	49
	Shrimp Shell Powder	15.6	Demineralized Shrimp Shell Powder	34
	Prawn Shell Powder	31	Demineralized Prawn Shell Powder	65.1
	Squilla Shell Powder	8.3	Demineralized Squilla Shell Powder	21.3
	Squid Bone Powder	2.4	Demineralized Squid Bone Powder	81
Pure Substrates	Crab Shell Chitin (Win Lab. Uk)	72	Crab Shell Chitosan 70% Deacetylated	35.1
	Crab Shell Chitin	61.4	Crab Shell Chitosan 100% Deacetylated	27.6
	Shrimp Shell Chitin	54	Microcrystalline Cellulose (Avicel)	2.5
	Prawn Shell Chitin	85.2	CMC	8
	Squid Bone Chitin	123	Xylan	11.3
	Calmar Pen Chitin (Crude)	127.2	Pectin	0
	Shrimp Shell Colloidal Chitin	100	Starch	0
	Crab Shell Swollen Chitin	64.2	Dextran	0
	Crab Shell Chitosan (Sigma USA)	45.3	Agarose	0

3.2. Effect of shelf-life stability on chitinase activity

As described in Material and Methods the chitinase activity shelf life stability was determined in three different temperatures - 200 4 and 25ºC. Data recorded and graphically represented in Fig. (1) showed that the temperature -20ºC was the most appropriate temperature for chitinase storage where the enzyme exhibited a relative resistance to freezing and thawing conditions ,where its live 4 cycles (4 weeks) with missing 30% from the initial activity. On the other hand, at the temperatures 4 and 25ºC the enzyme retained 43.6% and 44.9% of its initial activity after the first month and 12% and 27.6 after the second month. Bhushan and Hoondal 1998[24] found that shelf life of the chitinase of *Bacillus sp*. BG11 was 2 months at 4ºC using 10mg/ml sodium azide as preservative. In addition Bhushan (2000) [25] showed also that chitinase exhibited a shelf life of 4 weeks at 25ºC in addition of sodium azide, sodium metabisulphite, potassium chloride and glycerol, where as three-quarters of the initial activity has been conserved more than to 2 months in the four additions and the enzyme survived 10 freezing and thawing without any lost in activity, and Abdel Fatah and Khella (1995) found that chitinase of Streptomyces cellulosae F2 lost about 60% of its activity after the 7 days storage at room temperature[26] .

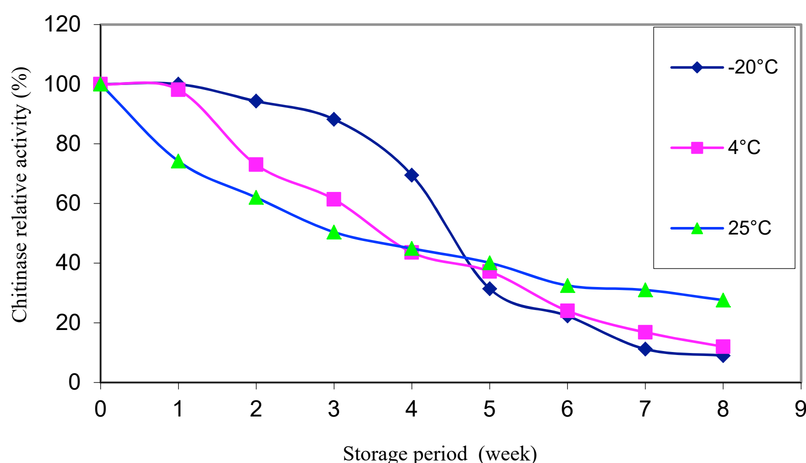

Fig. 1. *Bacillus Sp. R_2* Chitinase shell-life stability.

3.3. Chitinase anti-fungal activity

Fig. (2) indicated that the chitinase showed a moderate anti- fungal activity against many phytopathogenic fungi. The highest chitinase activity was obtained from the hydrolysis of Aspergillus niger mycelium followed by *Penicillium degitatum, Aspergillus flavus, Fusarium calmorum* then *Penicillum sp*. and finally *Macrophomira sp*. and *Rhizoctonia solani.* These results were in conformity with that reported about the anti fungal activity of the bacterial chitinases for example, the chitinases preparation of *Bacillus circulans* hydrolyzed *Aspergillus oryzae* and *Pyricularia oryzae*[27] , *B. cereus* UW85 hydrolysed *phytophtora medicaginis*[28] , *P. nicotianae*[29] , *Pythium aphanidermatum*[30] and *Sclerotinia minor*[31] *Bacillus sp.* chitinase antagonist *Gaeumannomyes graminis* [32]. On the other hand, Lee et al. (2000) [33] found that purified chitinase of Pseudomonas sp. YHS2 inhibited the growth of Fusarium oxysporm, Botrytis cinera and *Mucor rouxii*, Jung et al. 2003[34] found that the chitinase of *Paenibacillus illinoisensis* KJA-424 antagonist *Rhizoctonia solani*, while El Sayed et al. 1999[35] found the chitinase of *Streptomyces albovinaceus* S-22 hydrolysed the mycelia of *A. niger, F. oxysporum F. lycopersici*, *Trichoderma sp*. and *A. flavus*.

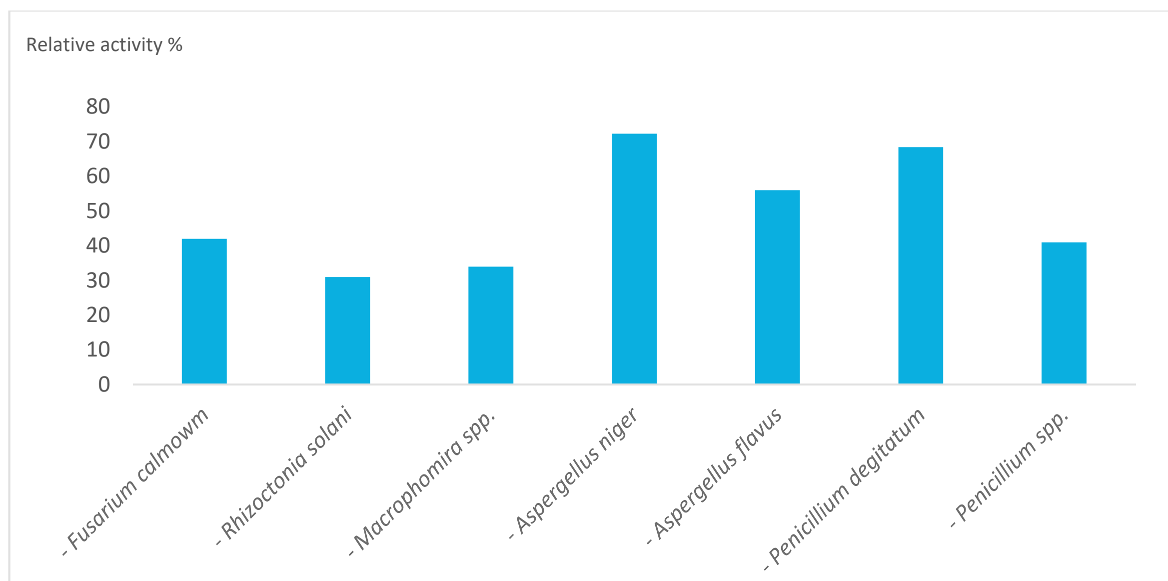

Fig. 2. antifungal activity of *Bacillus Sp. R2* Chitinase enzyme.

4. Conclusion

We conclude from chitinase substrate specificities and antifungal studies, thus more information can be obtained for understanding, chitinases characteristics/ functions relationship which is very important for developing of functional chitinases that meets specific biotechnological uses such as pharmaceutically important chitooligosaccharides preparation or promising antifungals production.

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