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Immobilization, Thermodynamic studies and Application of Chitinase enzyme from *Penicillium chrysogenum*

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ABSTRACT

This study was conducted to investigate the production of an efficient chitinase from a marine isolate. Accordingly, a marine fungus was isolated from the soft coral; *Heteroxenia fuscescens* and identified by molecular 18S rRNA technique as *Penicillium chrysogenum*. The isolate was chitinase producer (331, 8 U/mL) and the result was confirmed by TLC and HPLC. The enzyme was partially purified and immobilized completely in a modified bentonite. The free and immobilized enzymes recorded a maximum relative activity (100%) at 50 and 55°C, respectively, the activation energy of the immobilized enzyme was 10.60 Kjmol $^{-1}$ with 40% reduction than that of the free enzyme. Thermal and pH stability studies showed that the enzyme maintained its complete activity at (50 and 55) °C for 120 min and at pH 5 and 6. The deactivation rate constant (K_d), deactivation energy (Ed), half-life ($t_{1/2}$) , D, Δ G, Δ H, Δ S values were calculated for both enzyme forms and insured the success of the immobilization process in enzyme protection. The immobilized enzyme was reused for three cycles without loss in activity. Toxicological studies of chitinase against 3rd instar larvae of *Cx. pipiens* L revealed high mortality rates.

INTRODUCTION

Marine environment considered as an important habitat to different endophytic fungi which classified as obligate or facultative. Marine-derived fungi can grow and sporulate in marine organisms (Kohlmeyer and Volkmann-Kohlmeyer, 2003; Li and Wang, 2009). They can be considered as a great reservoir of medicinal and industrial biologically active metabolites. Harsh conditions such as salinity, high pressure, oligotrophic conditions, extreme pH, low temperature, mineral content in seawater, and special lighting conditions share to the differences between the enzymes produced from the marine microorganisms and homologous enzymes from terrestrial microorganisms (Passarini *et al*., 2011; Rämä *et al*., 2014).

Chitinases (E.C 3.2.2.14) known as glycosyl hydrolases had sizes ranged from 20 kDa to about 90 kDa (Bhattachrya 2007). The chitin degrading enzymes act

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directly in breaking down 1→4 β-glycoside bond in chitin to produce monomers and oligomers of N-Acetyl glucosamine (NAG) (Howard *et al*., 2003). Chitinases have wide applications in industrial, agricultural, and medical functions. They played a significant function in human health care, especially in human diseases as asthma and the preparation of pharmaceutically important chitooligosaccharides] and N-acetyl D glucosamine (Hakala *et al*., 1993) also, the preparation of single-cell protein, isolation of protoplasts from fungi and yeast, control of pathogenic fungi, treatment of chitinous waste, mosquito control and morphogenesis (Hamid *et al*., 2013). Moreover, chitinases were used in immunosuppressed patients as detectors for fungal infections (Lupetti *et al*., 2011).

Bacteria, plants and fungi are the most abundant sources for chitinases (Hamid *et al*., 2013).

Fungi are able to produce chitinases which degrade chitin for survival. During the development of the fungus, chitinases could breakdown the old cell wall chitin for the formation of a new one (Dahiya *et al*., 2006). Due to this mechanism, these enzymes were used as biological agents that could fight fungal pathogens. Some organisms (*Trichoderma, Bacillus, Rhizobium and Streptomyces*) have the ability to produce antifungal chitinases (Benitez *et al*., 2004).

Chitinolytic enzymes were used as supplements for chemical fungicides to increase their effectiveness against pathogenic molds and reduce the required concentrations of these harmful chemicals (Singh *et al*., 1999).

Chitinase immobilization was used for the hydrolysis of chitin, pH and thermal stability, catalytic activity and enzyme reusability (Longo *et al*., 1992). The most important issue takes a part in enzyme immobilization is the selection of the proper carrier which could be reuse efficiency for several times. Also, the carrier low cost is urgent demanded for industrial application (Esawy *et al* 2016). Recently, the enzyme thermodynamic study takes a part in many researches due to its influence in evaluation of the enzyme thermal stability (Karam *et al*., 2017). Gibbs free energy (Δ G), enthalpy (Δ H), (entropy) Δ S are the most important parameters which could judge the enzyme ability to tolerate the temperature elevation (Mostafa *et al*., 2018).

One of the known biocontrol agents was the Entomopathogenic fungi which played a major role in insects and pests' control (Fang *et al*., 2012). *Cx. pipiens* L. complex of mosquitoes was established a long time ago but it was regarded as a global problem since 1960s. Mosquitoes of this species are widely distributed and are of veterinary and medical importance (Vinogradova 2003). The success of the *Cx. pipiens* mosquitoes is partly due to their ability in exploitation of the large amounts of food found in standing water sources generated by humans and livestock. Unlike most other species of mosquitoes, *Cx. pipiens* commonly breeds in aquatic habitats with a high organic content (Farajollahi *et al*., 2011). In Egypt, it was reported that lymphatic filariasis (Harb etal., 1993), Rift Valley fever virus (Hoogstraal *et al*.,1989), and West Nile virus (Soliman *et al*., 2010) were transmitted by *Cx. pipiens.*

Till this time, there is a rare study on *Penicillium chrysogenum* chitinases, also according to our knowledge none reported in the effect of free and immobilized chitinase in 3rd instar larvae of *Cx. pipiens* L. In this study we investigated the production of chitinase enzyme from marine derived isolate which was identified as *Penicillium chrysogenum.* The enzyme was partially purified and immobilized on Bent-CTAB by different methods. The adsorption technique was selected as the most proper method. Comparative study was achieved between the free and the immobilized enzymes. Finally, applicable experiment was done to evaluate the effect of the two enzyme forms on 3rd instar larvae of *Cx. pipiens* L.

MATERIALS AND METHODS

Microorganism

The fungus was isolated from the soft coral; *Heteroxenia fuscescens*. Marine isolate was maintained in starch agar medium which had the following components (g/L) : Starch, 10; yeast extract, 4 ;peptone, 2 ; agar ,20 in 1L of marine water adjusted at pH 7.2. The culture medium was incubated at 28^{o}C for 7 days (Nath and Smitha, 2015).

Isolate identification by 18S rRNA

The isolated endophyte was genetically identified according to Sigma Scientific Services Company method as follows: DNA extraction was made by Quick-DNA™ Fungal/Bacterial Microprep Kit (Zymo research #D6007). Then PCR clean up to the PCR product was made by using Gene JET™ PCR Purification Kit (Thermo K0701). Finally, the sequencing was made to the PCR product on GATC Company with ABI 3730xl DNA sequencer by using forward and reverse primers. Comparisons with sequences in the GeneBank database were achieved in BLASTN searches at the National Center for Biotechnology Information (NCBI) site (http://www.ncbi.nlMnih.gov).

Inoculum preparation

The spore suspension (3.2×10^{6}) cells/mL of the tested microorganism was transferred to 50 mL inoculum medium with the following ingredients (g/L): starch, 10; yeast extract, 4; peptone, 2 in 1L of marine water at pH 7.2, 200 rpm and 30°C for 3 days. A fixed volume of the inoculum (3 mL) was transferred to the fermentation medium (Nawani and Kapadnis , 2004).

Fermentation medium

This medium contained the following nutrients (g%) : $(NH_4)SO_4$, 0.7; K_2HPO_4; 0.1; $MgSO_4$, 0.01; yeast extract ; 0.05; NaCl, 0.1; Tryptone, 0.1; colloidal chitin, 0.5 in 100 mL of marine water at pH 7.2 . (Yuli *et al.*, 2004)

HPLC analysis of N-Acetyl glucosamine

Determination of N-acetyl glucosamine was performed by using of (Agilent, USA) High-performance liquid chromatography (HPLC) equipped with quaternary pump , refractive index detector, and shim-pack SCR-101N (300 mm L. x 7.9 mm l.D., 10 µm). The mobile phase was deionized water, degassed under vacuum in an ultrasonic bath. The flow rate was 0.7 mL min^{-1} at a temperature of 40°C. The quantification was achieved by comparison with analytical curves using standards. For TLC analysis, the product was chromatographed twice in a mobile phase containing n-butanol: methanol: 30 % ammonium solution: H_2O (5: 4: 2: 1) (v/v). Sprayed with aniline diphenylamine reagent and baking at 121 °C for 5-10 min (Suginta, 2007).

Exochitinase and protein assays

N- acetyl hexosaminidase activity was determined by the following method (coudron *et al*., (1998); 50 µL of p-Nitro phenyl –β-N-acetylglucosaminide (1mg/ mL of 0.05 M acetate buffer at pH 5) was mixed with 50 µL of crude enzyme and incubated at 30°C in shaking water bath for 1 hour. The reaction was stopped by the addition of 2.5 mL of 0.125 M sodium borate buffer (pH 10.). The amount of the released p-nitro phenol was measured at 410 nm. One unit of the enzyme activity was defined as the amount that releases 1 µmol of p-nitro phenol per min. The content of protein was also determined (Lowery *et al*. 1951).

Preparation of Cetyltrimethylammonium bromide (CTAB) – Bentonite

Bentonite powder (5 g) was dispersed in 500 mL hot water. Cetyltrimeth-ylammonium bromide (CTAB) 0.25 g was dissolved in 100 mL hot water and slowly

dropped into the bentonite, stirred at 70 °C for 12 hrs., treated for one hour at 70 °C using ultrasonic bath and left to cool at room temperature overnight. The precipitate was filtered and washed several times with hot deionized water until no bromide ions were detected by silver nitrate solution. The obtained clay was dried in an oven at 60 °C for 12 hours and ground in a mortar to obtain fine powder.

Partial purification and immobilization of exochitinase

Enzyme precipitation was performed by increasing the volumes of cold acetone (30, 40, 50, 60, 70, 80 or 90) % which were added slowly to an ice cold-enzyme solution until the acetone required concentration was reached. After centrifugation, the precipitated fractions were removed, dried and each enzyme fraction was assayed for exochitinase activity and protein content. The partially purified enzyme (0.02 g) was dissolved in 0.1 mL of 0.1 M phosphate buffer at pH 5 and added to 0.05 g of modified Bentonite which was previously shaken with 0.25 mL of 0.1 M phosphate buffer (pH 5.0) at room temperature for 15 min. The mixture was shaken for 24 hrs. at 30°C, washed with 2 mL distilled water and centrifuged.

Characterization of the free and immobilized Chitinase

Optimum temperature and pH

P-Nitro phenyl –β-N-acetylglucosaminide (50 µL) was mixed with 50 µL of crude enzyme or 0.03 g of immobilized enzyme and incubated at the following range of temperatures; 30, 35, 40, 45, 50, 55 and 60 °C. To investigate the effect of pH on the chitinase activity, 0.01g of the free chitinase and 0.03 of the immobilized chitinase were dissolved separately in 0.5 mL buffer solutions that cover the range of pH from (4-10) for one hour. The absorbance of the reaction mixture was read at 410 nm and the activity of the enzyme was calculated.

The activation energy (Ea) of catalysis for both the free and immobilized amylase forms was determined from the slope of theArrhenius plot [log V (logarithm of % residual activity) versus recip-rocal of absolute temperature in Kelvin (1000/T)], which is given by the following Eq. (2) (2) where R is the gas constant (8.314 mol−1k−1).Slope = −Ea R

Thermal and pH stabilities.

Before the activity of the free and immobilized enzymes has been evaluated, both examined enzymes were preheated at elevating levels of temperatures; 50, 55, 60, 65 and 70°C for different time intervals (0.5, 1, 1.5 and 2) hrs. The stability of the activity at different pH was assessed by exposing the enzymes to a range of pH values from 4 to10 for 30, 60, 90 and 120 min.

Impact of salts on enzyme potency

The following salts; CaCO3, $MgSO_4$, $FeSO_4$, KCl, EDTA, $NaNO_3$, KI, NaCl, $(NH_4)_2SO_4$, $CuSO_4$ and $KHCO_3$ were prepared to reach 2mM final concentration. The free enzyme (0.01 g) and the immobilized enzyme (0.03g) were dissolved separately in 0.5 mL of phosphate buffer at pH 5 for free and at pH 6 for immobilized enzymes. and incubated with 0.5 mL of each salt for one hr. at room temperature before the activity has been calculated.

Thermodynamic study

The k_d was estimated by regression plot of log relative activity (%) versus time (min). The $t_{1/2}$ and *D-value* for *A.niger* exochitinase were determined from the Eq. (3,4)

$$t_{1/2} = \frac{ln2}{k_d} \quad (3)$$

$$D - value = \frac{ln10}{k_d} \quad (4)$$

The activation energy (E_d) for exochitinase denaturation was determined by a plot of ln denaturation rate constants ($ln\ k_d$) versus reciprocal of the absolute temperature (K) using the Eq. (5)

$$Slope = -\frac{E_d}{R} \quad (5)$$

The change in enthalpy ($\Delta H°$, kJ mol^{-1}), free energy ($\Delta G°$, kJ mol^{-1}) and entropy ($\Delta S°$, J mol^{-1} K^{-1}) for thermal denaturation of exochitinase were determined using the

following Eq. (6, 7, 8)

$$\Delta H° = E_d - RT \quad (6)$$

$$\Delta G° = -RT\ ln\left(\frac{k_d.h}{k_B.T}\right) \quad (7)$$

$$\Delta S° = \frac{\Delta H° - \Delta G°}{T} \quad (8)$$

Where T is the corresponding absolute temperature (K), R is the general gas constant.

Operational stability

The ability of the enzyme to be reused as a biocatalyst for the hydrolysis of p-Nitro phenyl –β-N-acetylglucosaminide was investigated for five runs. After the end of each run, 0.03 g of the immobilized enzyme was washed with distilled water and prepared for the next run and the activity of the released enzyme was calculated.

Statistical analysis

Data analysis was carried out with MICROSOFT EXCEL (2007). All data are presented as means ± standard error of means. The experiments were replicated 3 times (n = 3), with 3 measurements per replicate. The mean of the repeated measurements yielded the value for each replicate.

Toxicological studies of chitinase on mosquito larvae, Culex pipiens L.

Laboratory maintenance of Culex pipiens L.:

Egg rafts of the mosquito, *Cx. pipiens* were obtained from the Research and Training Center on Vectors of Diseases, Ain Shams University, Cairo. The sample was reared for at least four generations in an insectary in Entomology Department, Faculty of Science, Ain Shams University, Cairo under laboratory conditions (27±2ºC, RH 70±10%, and 14-10 hrs. light-dark regime). Egg rafts were placed in white enamel dishes filled with 1500 mL of distilled water. Newly hatched larvae were fed on fish food (Tetra Min, Germany) as a diet (Gerberget. 1970, Kasap and Demirhan ,1992).

Stock Suspensions of the free and immobilized enzymes

Stock suspension of free enzyme was prepared by suspending 0.8 g of free enzyme in 80 mL of 20 % Triton x-100. Stock suspension of immobilized enzyme was prepared by dissolving 0.7 gm of immobilized enzyme in 70 mL of 20% Triton x-100.

Toxicological evaluation:

The larvicidal activity of free and immobilized enzymes was evaluated against the late third instar larvae of *Cx. Pipiens* under laboratory conditions (27±2ºC, RH 70±10%, and 14-10 light-dark regime). Bioassay tests were carried out according to

the standard World Health Organization larval bioassay test method (WHO 2005). Batches of twenty late third instar larvae were transferred by a plastic dropper to three small disposable test cups, each containing 100 mL of water. Tested enzymes were applied by immersion method; the tested concentrations 0.01, 0.03, 0.05, 0.08, 0.1, 10 and 100 ppm were prepared and added to the cups. The untreated (control) test was performed for each concentration using the corresponding concentration of Triton x-100.

Three replicates were performed for each concentration and the untreated test. After 24 and 48 hrs. exposure, larval mortality was recorded. Moribund larvae were counted and added to dead larvae for calculating percentage mortality.

Calculations and data analysis:

Mortality percentages of larvae were calculated and corrected for natural mortalities (Abbott, 1925).

Concerning the relative susceptibility of 3rd instar larvae to the tested enzymes, the obtained results were translated to probit-log concentrations. Computed percentage of mortality was plotted versus the corresponding concentrations using Ldp line software program to obtain the toxicity regression lines. Percentages of corrected mortalities were statistically analyzed (Finney, 1972). The tested enzymes were compared for their efficiency on *Cx. pipiens* larvae according to their LC_{50}, LC_{90} and slopes of the toxicity lines.

The toxicity index was employed for the direct comparison of insecticides (Sun, 1950).

Toxicity index (Sun's equation) = LC_{50} of the most effective compound/ LC_{50} of the tested compound x 100.

Relative potency values were calculated as follows:

Relative potency = LC_{50} of the lowest toxic insecticide/ LC_{50} of the tested insecticide (Zidan and Abdel-Mageed 1988).

RESULTS

Isolation and molecular18srRNA identification of the marine strain

The studied fungus was isolated from the soft coral *Heteroxenia fuscescens.* The sample was collected from the depth of the Red sea (Hurghada). The fungus was subjected to 18S rRNA (Ribonucleic acid) sequencing technique which was performed to identify the fungal isolates on a molecular level. The phylogenetic tree (Fig. 1) showed that the active strain was most closely related to *Penicillium chrysogenum* strain F-99, small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1,5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene. The fungus was submitted to the Genbank but the similarity of the sequencing and multiple alignments confirmed that the fungus was in a close relation to *Penicillium chrysogenum* strain MF077263.1 (99% identity).

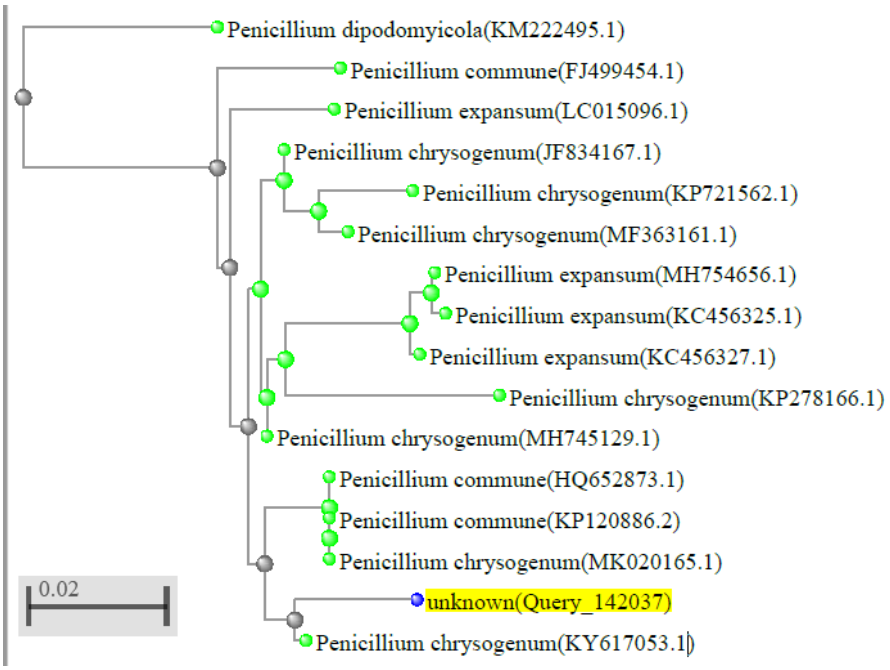

Fig. 1Phylogenetic tree based on the DNA nucleotide sequence of the 18S rRNA gene of the marine isolate

TLC and HPLC analysis of N-Acetyl glucosamine

The results of TLC chromatogram showed the presence of N-Acetyl glucosamine spot and its maximum concentration was observed after 6 hrs. of enzyme incubation with the substrate and it was disappeared completely after 24 hrs. HPLC analysis was done to confirm the previous result. Accordingly, the enzyme was incubated with the colloidal chitin for 6 hrs. and their products were analyzed by HPLC. As shown in Fig. 2, the production of the (N-acetyl glucosamine) was detected at 7.2 min which represented the same detection time of the standard N-acetyl glucosamine.

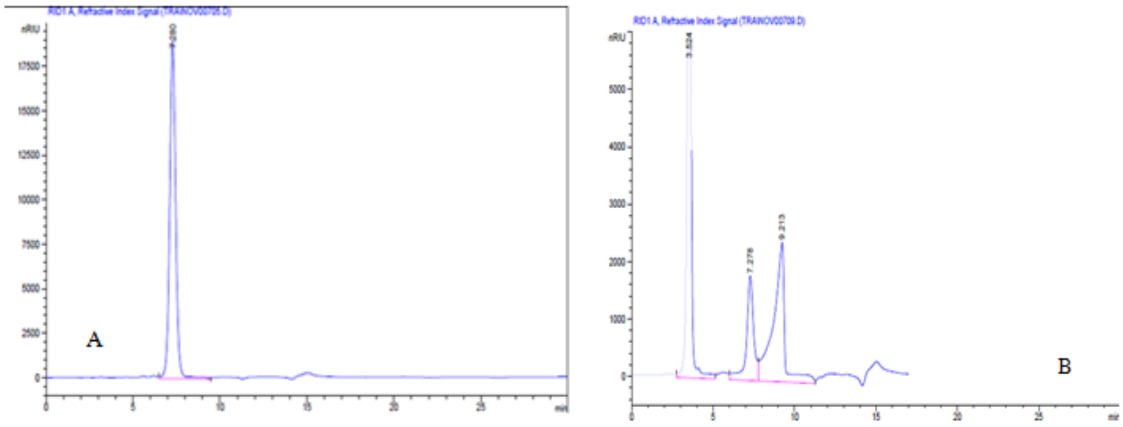

Fig. 2: HPLC result of standard N-acetylglucosamine (A).HPLC picture of the reaction mixture after 6 hours (B).

Immobilization of the partial purified enzyme

The enzyme was partially purified and the most active fraction was obtained at 30% acetone. The enzyme showed 10.5 purification fold. The purified form was immobilized by two methods adsorption and covalent binding on Bentonite+GA or Bentonite+ Epi. The enzyme adsorbed on Bentonite only showed complete immobilization yield (100%) followed by Bentonite+GA and Bentonite+ Epi (47 and 29 % respectively) as shown in Table 1.

Table 1: Immobilization of partially purified exochitinase on clay (Bentonite) by the use of Glutaraldehyde (GA) and epichlorohydrin (EPi)).

Carrier	Enzyme added (U/g carrier)	Protein (mg/ml) ???	Sp. activity	Unbound Enzyme (U/g carrier)	Protein (mg /ml) ???	Immobilized Enzyme (U/g carrier)	Protein (mg /ml) ???	Sp. activity	Immobilization Yield (%)
Bentonite	480.6	0.22	2181	164	0.18	330	0.04	8250	100
Bentonite+ **GA**	440.8	0.23	2086	38	1.39	188.8	0.1	1888	46.9
Bentonite+ EPi	440.8	0.23	2086	5.8	0.23	123.6	0.11	1123	28.4

I/A-B

Influence of temperature and pH

The results in Fig. 3A showed that the highest relative activities were obtained at 50 and 55 ^{o}C for the free and the immobilized enzymes, respectively (Fig. 3A). Furthermore, in compared to the free enzyme, the immobilized chitinase was more tolerant to a wide range of high temperatures (45 to 60^{o}C). Also, the activation energy was calculated for the free and the immobilized form to record 17.46 KJ·mol^{-1} and 10.55 KJ·mol^{-1} respectively (Fig. 3B).

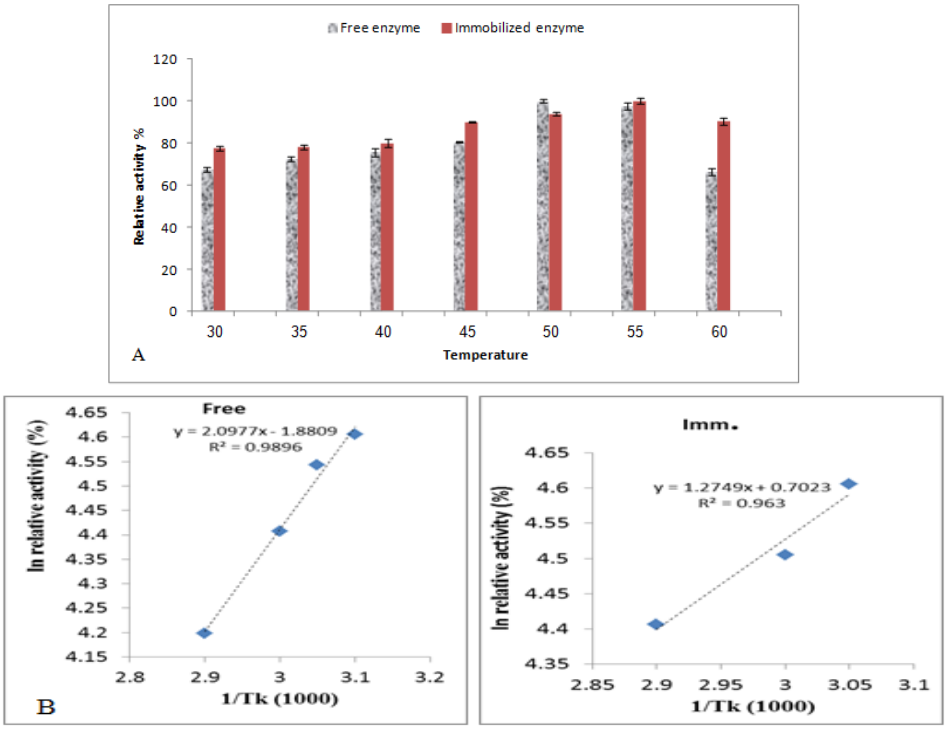

Fig. 3: Effect of temperature on free and immobilized *Penicillium chrysogenum* chitinase (A). Arrhenius plot for activation energy (Ea) (B).

The free and immobilized enzymes recorded maximum activity; (100%) at pH 5 and 6 respectively, also a slight reduction was observed for free enzyme at pH 4, 6 (91.9 and 98.9% respectively). The alkaline pH led to drastic lost in enzyme activity where the free enzyme lost its activity completely at pH 10 (Fig.4).

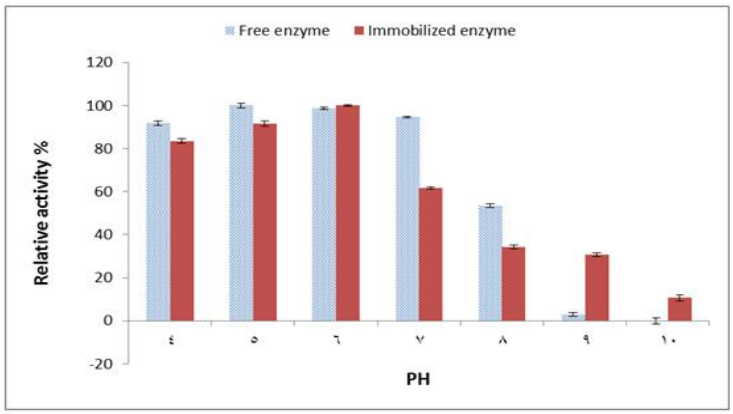

Fig. 4. Effect of the reaction pH on the free and immobilized *Penicillium chrysogenum chitinase*

Thermal and pH stability

The thermal stability study recorded 100% stability for the free and the immobilized form at 50-55ºC and different time intervals (30-120 min.). At 65-70 ºC the free enzyme approximately lost its activity completely, while the immobilized one could keep 33-24% of its original efficacy (Fig. 5).

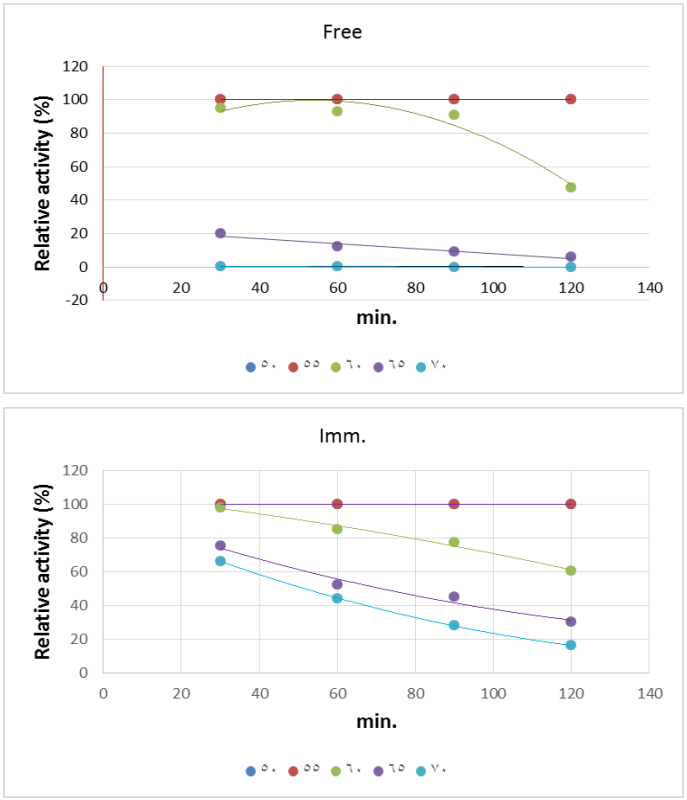

Fig. 5: Thermostability profile of the free and immobilized enzyme

The pH profile for both enzyme forms recorded a complete stability at pH 5, 6 at the different times intervals, except 13% loss of free enzyme original activity at pH

6 after 120 min. Also, the free and the immobilized forms tolerated the pH 4 and maintained about 77-62 % of their activities. The alkaline pH had a great adverse effect on the free enzyme and sharp decrease in activity was noticed, while the immobilized form was more tolerant to the alkaline effect than the free chitinase Table 2.

Table 2: pH stability of the free and immobilized chitinase from.

pH	Relative activity (%)							
	Free enzyme				Immobilized enzyme			
	Exposure time (min)							
	30 min	60 min	90 min	120 min	30 min	60 min	90 min	120 min
4	77.4	69.7	65.9	55.4	69.5	68.9	65.9	62.8
5	100	100	100	100	100	100	100	100
6	100	100	100	78.8	100	100	100	100
7	71.4	67.3	54.4	17.0	54.1	41.2	38.8	34.7
8	42.8	38.8	37.6	29.1	23.6	23.6	23.3	22.1
9	5.40	1.10	0.70	0.48	20.5	17.2	14.0	8.60
10	0.00	0.00	0.00	0.00	12.1	10.7	10.3	6.9

Thermodynamic studies

Some thermodynamic parameters were evaluated such as activation energy for denaturation (Ed), half-lives $T_{1/2}$ and D-values (decimal reduction time). Ed value for the free and immobilized form were determined to be 97 kJ mol^{-1} and 101 kJ mol^{-1} (Fig. 6). The The $t_{1/2}$ for the free and the immobilized form recorded 230, 138, 77 min. and 345, 172, 115 min respectively. The D value for both enzyme forms were 766, 460, 255 min. and 1150, 575, 383 min. In Fig. 7. first order of thermal deactivation (K_d) of free and immobilized *Penicillium chrysogenum* chitinase was determined. The result showed that the K_d of the immobilized enzyme was lower than the free enzyme. For instance, at 50 ºC the K_d of the free and immobilized were 0.0031 0.0022 min^{-1} respectively.

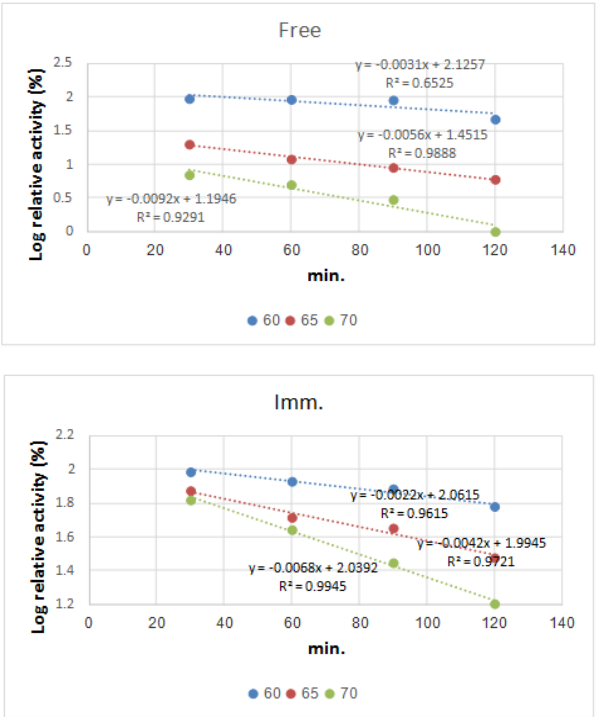

Fig. 6. First order of thermal deactivation of free and immobilized *Penicillium chrysogenum* chitinase.

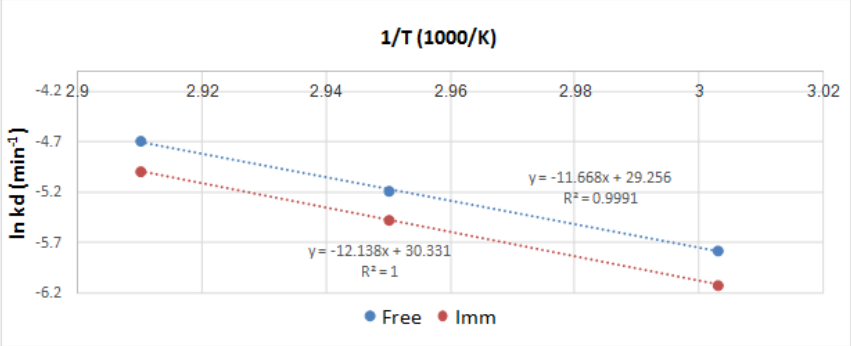

Fig. 7: Arrhenius plot to calculate activation energy for denaturation free (E_d) and immobilized enzyme.

The ΔH° values were 72.033, 72.47, 72.8 for the free form and 76.033, 76.47, 76.8 for immobilized form respectively. While the ΔG° was calculated as 765, 736, 714 and 737, 743, 721 for free and immobilized form respectively. ΔS° recorded negative results in both the free and the immobilized enzyme (Table 3).

Table 3: Thermodynamic parameters of free and immobilized chitinase isolated from *Penicillium chrysogenum* **.**

Thermodynamic parameters	Free	Immobilized
Ed (kJ mol^{-1})	97	101
$t_{1/2}$ (min)		
60°C	230	345
65 °C	138	172
70 °C	77	115
D-value (min)		
60°C	766	1150
65 °C	460	255
70 °C	575	383
ΔH° (KJ·mol−1)		
60°C	72.033	76.033
65 °C	72.47	76.47
70 °C	72.8	76.8
ΔG° (KJ·mol^{-1})		
60°C	765	737
65 °C	736	743
70 °C	614	731
ΔS° (KJ·mol^{-1})		
60°C	-231	-220
65 °C	-224	-226
70 °C	-220	-222

The role of added salts on the capacity of the enzymes

The results in Table 4 confirmed a reverse effect of all tested salts on the efficiency of the free enzyme where the enzyme lost nearly 50% of its activity. On the contrary, the immobilized enzyme was activated by the addition of KI, $CuSO_4$, $(NH_4)_2SO_4$ and $CaCO_3$, where the relative activity was shown be 132.4,130,121 and 113.6 % respectively. Also, a slight decrease in activity was noticed by EDTA, KCl and $KHCO_3$, respectively.

Table 4: Effect of different salts on the activity of free and immobilized chitinase from *Penicillium chrysogenum*

Salt	Relative activity (%)	
	Free	Immobilized
Non	100	100
$CaCO_3$	49.2	113.6
$MgSO_4$	56.6	105.2
$FeSO_4$	41.6	98.3
KCl	54.3	91.0
EDTA	55.1	87.4
$NaNO_3$	56.6	109.5
KI	43.7	132.4
NaCl	45.8	103.3
$(NH_4)_2SO_4$	59.7	120.6
$CuSO_4$	31.2	129.8
$KHCO_3$	48.0	95.0

Operational stability of immobilized chitinase

The results in Table 5 confirmed that the immobilized enzyme could be reused for three times with a complete activity. At cycle No 4 the enzyme lost 50% of its original activity and a complete loss of enzyme activity was obtained at cycle No 5.

Table 5: Toxicological evaluation of free chitinases after 24 and 48 h

Toxicological evaluation	Mortality after 24 h.	Mortality after 48 h.
slope	0.452±0.0384	0.4396±0.0394
Chi-Square (χ^2)	7.99 (tabulated 11.1)	4.4384 (tabulated 11.1)
Correlation Coefficient(r)	0.9746 (tabulated 0.755)	0.9859 (tabulated 0.755)
LC_{50} (Its limits at 95%)	3.9745 (2.1741-8.2948)	0.5384 (0.3191-0.9591)
LC_{90} (Its limits at 95%)	2720.886 (700.5991-17521.29)	443.0039 (129.7105-2448.476)
Toxicity index	100	100
Relative potency	5.690	11.3921

Toxicological Evaluation

The potency of the two tested enzymes, free and immobilized chitinases was evaluated against the late 3rd instar larvae of *Cx. Pipiens*as (Fig. 8 A and B). Median lethal concentration (LC_{50}) was calculated using Ldp line software. Data presented in Tables 6 and 7 indicated that the highest mortality rate was achieved by free chitinase which recorded the lowest LC_{50} values with 3rd instar larvae. Whereas, LC_{50} values for 3rd instar larvae of *Cx. pipiens* treated with free and immobilized chitinases after 24 hours were 3.974 and 27.754 ppm and after 48 hours were 0.538 and 5.635, respectively. On the other hand, $LC_{90,}$ values after 24 hours revealed 2720.886 and15483.16 ppm and after 48 hours 443.004 and 5046.777, respectively. The obtained data indicated that free enzyme was the most potent against *Cx. pipiens* larvae.

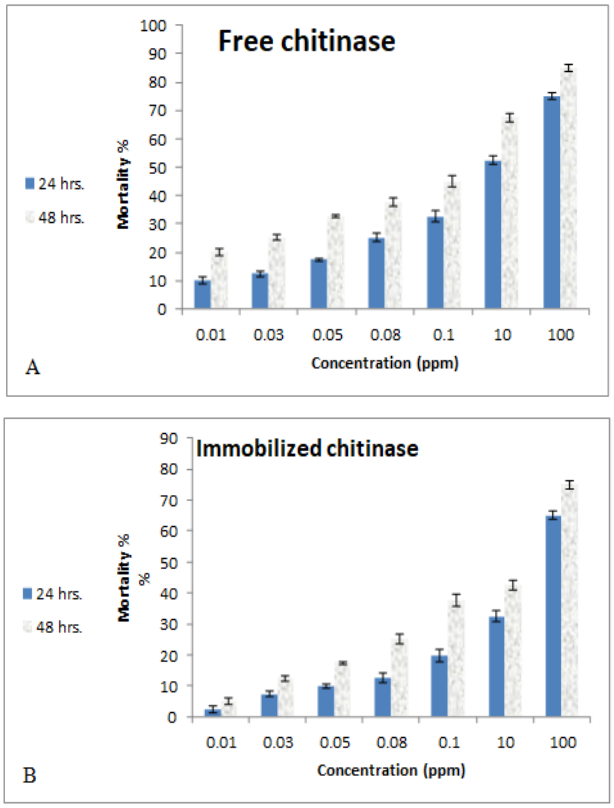

Fig .8: Toxicity results of free (A) and immobilized enzymes (B) on 3rd instar larvae of *Cx. Pipiens* after (24 and 48) h.

DISCUSSION

Marine environment is expected to carry microorganisms with unique properties. This is due to harsh and challenging conditions which push the microorganisms to adapt themselves for surviving. Many reports detected different types of potential chitinases from the marine environment (Paulsen *et al*., 2016, Krishnaveni and Ragunathan 2014). In this report, a marine derived fungus was found as a good chitinase producer (331,8 U/mL).This result was higher than Gomaa *et al*,(2011) who reported that the amount of *Bacillus thuringiensis* and *Bacillus licheniformis* chitinases were 25.36 U/mL and 23 U/mL. *Aspergillus awamori* EM66 exochitinase which showed 5.9 U/mL (Esawy *et al*., 2016) and lower than *Aspergillus flavus* chitinase 1400 µmoles of NAG/mL(Gunalan *et al*., 2012). The N-Acetyl glucosamine was detected by the use of TLC and confirmed by the HPLC analysis. The fungus was identified as *Penicillium chrysogenum* based on molecular18S rRNA sequence. Until this moment scarce researchs mentioned *Penicillium chrysogenum chitinase* (Sámi *et al*., 2001; Patidar *et al*., 2005).

The second step in this work started with partial purification of the enzyme with 30% acetone. The enzyme showed 10.5 purification folds where the recovered activity was 33.6%. The enzyme was immobilized on Bent-CTAB using the adsorption method and the covalent binding methods. The immobilization yield % of the adsorption method superior the covalent binding to great instant. This result could be back to the high porosity which make the enzyme diffusion into the bentonite easier and more efficient (Appelo 2013). It was also reported that the highest catalytic activity was noticed on Bent-CTAB-lipase (Dong *et al*. 2013). This resulted from the tunable hydrophilic/hydrophobic balance of the support's surface, since the excessive

hydrophobic property appeared negative influence on lipase's catalytic performance. The lower efficiency through the covalent binding by either GLD or PEI could be back to the conformation in enzyme active site during the reaction. It was worth to mention that the immobilization process increased the enzyme sp. activity about 3.7 times. This result could be explained that most of the *Penicillium chrysogenum* chitinase was immobilized on the Bent-CTAB and the undesired protein was escaped through the washing process. Comparative study was done to characterize the free and the immobilized enzyme. The results showed that both the enzyme forms were active in a wide range of temperatures with degree of superior in case of the immobilized form. Also, the maximum temperature for enzyme activity was converted from 50°C to 55°C after the immobilization. The shift in temperature might be back to molecular cage around the protein which had role in enzyme protein protection against the raise in temperature (Roger *et al*., 2004). Same observation was noticed with *Aspergillus awamori* EM66 exochitinase where the optimum temperature for the free and the immobilized enzymes were shifted from 35 to 45 °C (Esawy *et al*., 2016). The activation energy calculation is essential to evaluate the amount of energy which the enzyme required to be active. The study revealed a significance role of immobilization process in reducing the activation energy (Ea) of the enzyme where 17.44 Cal mol $^{-1}K^{-1}$ and 10.60 Cal mol $^{-1}K^{-1}$ were got for free and immobilized chitinase respectively. This result could be explained that the immobilization process managed the enzyme active site to combined more easily with the substrate or to the conformation in enzyme active site. Both enzymes were subjected to a wide pH ranges and the data illustrated a high resistance to the acidic medium with a slight decrease in activity. Furthermore, operational stability of the immobilized enzyme was performed and the enzyme was stable and active for three times, this result manages the chitinase to be used in industry due to its high stability. Usually, two essential kinetic parameters are often determined for an immobilized enzyme to evaluate the role of immobilization process on the enzyme's catalytic efficiency when compared to the free enzyme counterpart. They are the Michaelis constant Km and maximal reaction velocity. Km means the rate of enzyme -substrate binding while, Vmax measures the conversion rate of the substrate to product which called catalytic activity (Bommarius and Paye 2013).

Thermodynamic studies were done to evaluate the effect of the immobilization process in enzyme stability. The thermal stability profile showed complete stability for both enzyme forms at 50-55 ºC and insure the effect of the immobilization process in increment the *Penicillium chrysogenum* chitinase thermal stability from 60-70ºC. In general, $T_{1/2}$ and D-values noticeably decrease with the temperature elevation and the immobilization process take a significant role in $T_{1/2}$ prolongation. The results showed that the rate constant of inactivation (kd) for both free and immobilized enzyme progressively increased with temperature. Another important parameter could explain the enzyme thermal stability is the amount of energy demanded for the enzyme to start the denaturation process (Ed). This means that the inactivation process started and the thermo-stability was diminished. The results showed that the immobilized enzyme needs more energy for beginning the denaturation process in compared to its free form. The ΔG called Gibs free energy; it is a key factor for determining the enthalpy change Δ H and the entropy change Δ S. These parameters were evaluated such as , Δ G, Δ H, ΔS.The results referred to a slight increase in Δ H value after the immobilization process. This means that the immobilization process make the enzyme relatively more tolerant to the denaturation with the raise in temperature in compared to the free form (Mostafa 2018). Also, at

65ºC, 70 ºC the Δ G of the immobilized form exceeded the free form, this suggested that the immobilization process protected the enzyme against the denaturation process in compared to the free enzyme. In general, high Δ G accompanied with the low Δ H means that the enzyme was heat stable. The direction of Δ S to the negative side means that the enzyme was in ordered form (Nagar *et al* 2012).

All the previous result confirmed the success of the immobilization in increase the enzyme heat stability.

Insecticide applications, although highly efficient against the target vector species, are facing a threat due to the development of resistance to insecticides, other undesirable effects include toxicological effects against non-target organisms environmental and human health concerns (Liu *et al.*,2006).

The aim of the current work was to evaluate the efficiency of chitinase enzyme as insecticidal agents against the target insect vector.

In the present study, free and immobilized chitinases were used to evaluate their toxicological effects against 3rd instar larvae of *Cx. pipiens*. The tested enzymes revealed variations in LC_{50} and LC_{90} values. The obtained data cleared that the free enzyme was the most effective enzyme based on the lowest LC_{50} value followed by immobilized. The present results agreed with other repotrs (Cai *et al*.2007; Park *et al*., 2010).

CONCLUSION

This study aimed to compare between the efficiency of the free and the immobilized *Penicillium chrysogenum* chitinase. This was done through different points of aspects such as enzyme properties, kinetics and thermodynamic calculations. All the results pointed to the role of immobilization process in improving the enzyme efficiency and the ability of it to be reused for three times. Also, applied study was done to evaluate the effect of the enzyme in *Cx. pipiens* L. complex of mosquitoes. The results recommended *Penicillium chrysogenum* chitinase as a new insecticidal.

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