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Chitinase from *Bacillus subtilis*: Immobilization, antifungal activity and production of N-acetyl-D-glucosamine

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Chitinase was purified to homogeneity from *Bacillus subtilis* (GenBank accession no MN548732.1). The molecular weight of the purified chitinase was 52 KDa as determined by SDS-PAGE. Ethyleneglycol-bis(*β*-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) inhibited chitinase activity indicating that the enzyme is metalloenzyme. Chitinase was activated by thiol compounds such as CoA, cysteine and dithiothreitol (DTT). The cysteinyl, tyrosyl, histidyl and arginyl groups are essential for chitinase catalysis. Three carriers; calcium alginate, silica gel and amberlite were used as beads for immobilization of purified chitinase. Calcium alginate exhibited the highest immobilization yield compared to silica gel and amberlite. Co^{2+} and Mn^{2+} were activators of chitinase. The pH profile of free and immobilized enzyme was relatively stable over a wide pH range with optimal values at pH 5.0 and 6.0 for the two forms of enzyme, respectively. The optimal temperatures of free and immobilized chitinase were 50 ^{o}C and 60 ^{o}C, respectively. The immobilized chitinase displayed higher thermostability at 65 ^{o}C and 70 ^{o}C over the free enzyme. The K_m values for the free and immobilized chitinase were 1.7 and 2.7 mgml^{-1}. The V_{max} values were 143 and 294 Umg^{-1} protein, respectively. These findings indicate that the immobilization process can improve the enzyme properties. The purified chitinase expressed antifungal activity against *Aspergillus oryzae*, *Rhizoctonia solani*, *Fusarium solani*, *Fusarium oxysporum*, *Trichoderma reesei* and *Rhizopus oryzae*. The biodegradation of prawn shells and fish shell wastes by immobilized chitinase was employed for N-acetyl-D-glucosamine (NAG) production.

Keywords: Chitinase, *Bacillus subtilis*, Immobilization, Antifungal, N-acetyl-D-glucosamine.

INTRODUCTION

Chitin is a homopolymer of *β*-1,4-N-acetylglucosamine residues and it is found in two conformational structures (*α* and *β* chitin) depending on the polymeric chain arrangement. It signifies the second widely distributed bio-polysaccharides in nature. It is enormously distributed as a component of fungal cell wall, marine organisms, as well as the outer shell of arthropod and insect exoskeleton (Duo-Chuan, 2006; Karthik et al., 2014; Senol et al., 2014; Stumpf et al., 2019).

Chitinolytic enzymes are produced by various organisms as fungi, bacteria, actinomycetes, mammals and plants. It can perform several roles including morphological changes of cells, digestion of chitin and defence against phytopathogens (Van Eijk et al., 2005; Gohel et al., 2006; Singh et al., 2007; Rocha-Pino et al., 2011).

Chitinase belongs to the glycoside hydrolase family. It can be classified into exochitinases and endochitinases. Endochitinase (E.C. 3.2.1.14) is capable of catalyzing the random hydrolysis of

chitin polymer and release low chito-oligosaccharides. While, exochitinase catalyzes dual functionality reactions through action of chitobiase (E.C. 3.2.1.29) and N-acetylglucohexoaminidases (E.C. 3.2.1.52), generating N-acetylglucosamine monomers (Patil et al., 2002; Karthik et al., 2014; Farag et al., 2016; Alves et al., 2018).

Chitinase immobilization enables the hydrolysis of chito-oligosaccharides and the reusability for several cycles in the batch system for reducing the cost expense. Various techniques can be adopted in the immobilization process such as covalent, adsorption, entrapment, encapsulation in addition to cross-linking (Salman et al., 2008; Elnashar et al. 2009). The immobilized enzymes are preferred in industrial applications because it can act under harsh conditions and can be reused (Bai et al., 2006; Esawy et al., 2016).

Antimicrobial activity of chitinase is an important property in its application as biocontrol agent. Chitinase exhibited an inhibitory effect on fungi (Giridhar et al., 2012; Li et al., 2018). The chitinases with bifunctional biocontrol activity (antifungal and antibacterial) have been studied by Medeiros et al. (2018).

Fish industries generate solid wastes into the environment. Discarding of various solid wastes (20–60 % of fishery wastes) is a serious environmental hazard. These chitinous byproducts are applied for microbial growth and energy production. Chitinase was applied for biodegradation of various chitinous substrates producing N-acetyl-D-glucosamine (NAG) and chitio-oligosaccharide (Bozzano and Sarda, 2002; Das et al., 2019).

The objectives of the present work were to (i) purify chitinase from *Bacillus subtilis*, (ii) immobilize chitinase using various carriers, (iii) compare the biochemical characteristics of the free and immobilized enzyme and (iv) evaluate the possible use of chitinase against various fungal strains and production of N-acetyl-D-glucosamine.

MATERIALS AND METHODS

Microorganism and cultivation

Bacterial isolates were obtained from the soil rhizosphere according to the method of **Kumar et al. (2012)** and grown on nutrient agar medium containing % (Glucose 0.5; peptone 1.0; yeast extracts 0.5; NaCl 1.0; pH 7.0) for 24-48 h at 30 ± 1 °C. The culture was sub-cultured periodically on nutrient agar slants and maintained at 4 °C for extended periods of storage.

Phenotypic and genotypic identification of chitin degrading bacterial isolate

The most potent isolate producing chitinase (strain RBM) (Data not shown) was chosen and identified according to Bergey's Manual of Systematic Bacteriology (Holt et al., 1994). Subsequently, the bacterial genomic DNA was extracted from the isolates according to Yadav et al., (2009). The 16S rDNA amplification was carried out in PCR (polymerase chain reaction) using forward primer (5′-CAGGCCTAACACATGCAAGTC-3′) and reverse primer (5′-CGGGCGGTGTGTACAAG-3′) (Marchesi et al., 1998 and Osborn et al., 2000). PCR was done in 50 µL and applied as follows: denaturation for 4 min at 94 °C, 40 cycles of denaturation for 1 min at 94 °C, primer annealing for 30 sec at 62 °C, DNA extension at 72 °C for 1 min and a final 10 min extension at 72°C. The PCR products were separated on 1.2% (w/v) agarose gel electrophoresis, eluted and purified using the Qiaquick gel extraction kit (Qiagen, Germany) according to the manufacturer's instructions. The sequencing results were aligned and homology compared against nucleotide data bases retrieved from the GenBank databases with BLAST program that is available from the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov). Phylogenetic tree was conducted by CLUSTAL Omega based on the Neighbor-Joining method. The target strain was identified as *Bacillus subtilis* and the NCBI GenBank Accession No was MN548732.1 (Figure 1).

Preparation of colloidal chitin and chitinase production

The colloidal chitin (Sigma) was prepared according to Kuzu et al. (2012). *Bacillus subtilis* was grown on basal medium containing % (Na_2HPO_4 0.2; KH_2PO_4 0.1; NH_4Cl 0.1; NaCl 0.05; $CaCl_2.2H_2O$ 0.5; $MgSO_4$ $7H_2O$ 0.5 and 0.05% yeast extract at pH 7.0) complemented with 0.2% colloidal chitin and was incubated for 24 h at 37 °C using shaking incubator at 200 rpm.

Preparation of the crude chitinase extract

After incubation time (24 h at 37 °C) the culture was centrifuged at 9,000g for 20 min at 4 °C. The supernatant was filtered by filter paper (Whatman, Piscataway, NJ, USA).

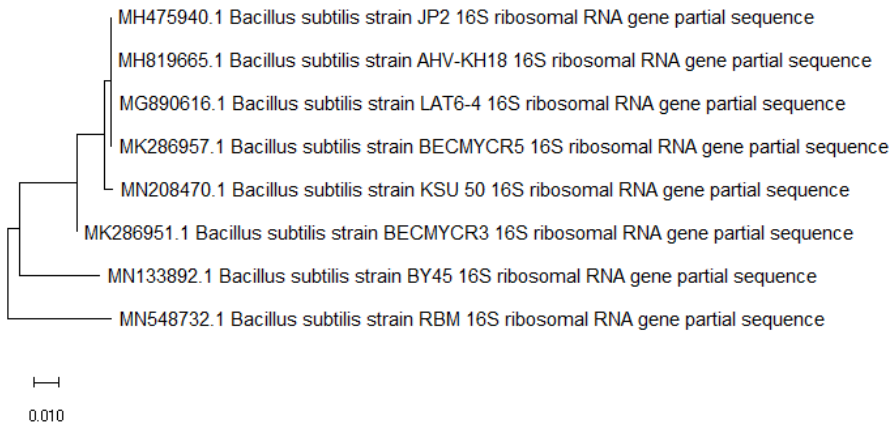

Figure 1: Phylogenetic tree based on 16S rDNA sequences of *Bacillus subtilis* RBM obtained from CLUSTAL Omega search representing the position of the target isolate and related strains.

The obtained supernatant was taken as the crude extract of the enzyme and saved at -20 °C for further work (Zarei et al., 2011).

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Assay of chitinase

The activity of chitinase was estimated by the modified method of Babashpour et al. (2012) with colloidal chitin (substrate). The assay medium contained 0.5 ml of chitinase and 0.5 ml of 0.2% colloidal chitin (w/v) in a phosphate buffer (200 mM, pH 5). The mixture was incubated for 30 min at 50 °C and then 3,5-dinitrosalicylic acid reagent (3 ml) was added followed by boiling for 5 min. The absorbance was measured spectrophotometrically at 530 nm and the standard curve prepared by N-acetyl-D-glucosamine (NAG). One unit of enzyme activity was termed as the amount of chitinase that generates one µmol NAG under standard assay conditions.

Protein determination

The determination of protein content was carried out by the method of Lowry et al. (1951).

Chitinase purification and molecular weight determination

The crude enzyme extract was precipitated by $(NH_4)_2SO_4$ powder (85% saturation) at 4 °C with gentle stirring for 1 h and centrifuged at 8,000×g for 25 min. The precipitate was dialyzed versus 100 mM potassium phosphate buffer (pH 7.0) for 24 h and used for estimation of chitinase activity as well as the protein content. The ammonium sulfate fraction was resulted after the dialysis was applied onto a diethylaminoethyl (DEAE)-Sepharose that was pre-equilibrated with 100 mM phosphate buffer, pH 5.0. The concentrated chitinase was loaded onto Sephadex G-200 column pre-equilibrated with acetate buffer (50 mM, pH 5.0). The elution of the bound protein was carried out using 50 mM acetate buffer (pH 5.0) in a stepwise gradient on 0.1-0.5 M NaCl. The active fractions with the highest enzyme activity were collected, pooled and concentrated against polyethylene glycol (PEG) 6000.

The molecular weight of the purified enzyme was carried out according to Laemmli (1970) using one SDS-PAGE.

Effect of different chemical compounds on chitinase activity

The activity of chitinase was determined in presence of chelating agent ethyleneglycol-bis(*β*-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), thiol compounds such as CoA, cysteine and dithiothreitol (DTT) and group reagents including 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) for cysteinyl, tetranitromethane (TNM) for tyrosyl, diethylpyrocarbonate (DEPC) for histidyl and 2,3-butanedione (BD) for arginyl (El-Shora and Metwally, 2008). Chitinase activity of the control without adding chemicals was assayed. The remaining activities were estimated by the standard assay.

Immobilization of chitinase

The purified enzyme was mixed in 1:1 ratio with sodium alginate (5% w/v) in 100 mM sodium phosphate buffer (pH 8.0). The mixture was dropped into 100 mM $CaCl_2$ solution with shaking at 4 ºC for 2 h. After filtration, the immobilized beads were washed with distilled water, then dried by Whatman filter paper No. 1 and stored at 20 ºC. The purified chitinase was also immobilized on silica gel and amberlite pre-equilibrated with phosphate buffer (100 mM, pH 8.0) and left at 4 ºC for 2 h. The unbounded chitinase was removed and the immobilized beads were re-suspended in 100 mM phosphate buffer (pH 8.0) (Prasad and Palanivelu, 2013; El-Shora et al., 2017).

Effect of pH and temperature on activities of free and immobilized chitinase on Ca-alginate

The optimal pH of the two forms of chitinase activity was assayed at pH range 2.0-9.0 using substrate solution of 0.2% colloidal chitin under standard assay conditions. The various buffers used were 200 mM sodium acetate (pH 2-6) and 200 mM phosphate buffer (pH 7-9). The influence of pH on the chitinase stability was tested by pre-incubating the enzyme in respective buffers for 40 min at room temperature. The remaining activity was estimated as mentioned before.

To investigate the influence of reaction temperature on the activities of the two forms of the enzyme. The reaction mixture was incubated at 20, 30, 40, 50, 60, 70 and 80 °C using 0.2% colloidal chitin as the enzyme substrate. For thermostability assay, each of the two forms of chitinase was pre-incubated for 80 min at 65 °C and 70 °C. The remaining activity was measured at regular intervals.

Effect of various metal ions on chitinase activity

The impact of various metal ions (Mg^{2+}, Mn^{2+}, Fe^{3+}, Hg^{2+}, Ca^{2+}, Cu^{2+}, Co^{2+} and Cd^{2+}) on free and immobilized chitinase was carried out in the presence of 5 mM chloride salt. Chitinase activity was assayed in the absence of metal ion and defined as 100%.

Determination of K_m and V_{max} of chitinase

Michaelis constant (K_m) and the maximum velocity (V_{max}) of the free and immobilized chitinase were determined by the Lineweaver-Bürk plot (Lineweaver and Bürk, 1934).

Assessment of the antifungal activity of the purified chitinase

The antifungal activity of the purified chitinase was studied by measuring the diameters of the inhibition zones according to Ye and Ng (2000). The investigated fungal isolates (*Aspergillus oryzae*, *Rhizoctonia solani*, *Fusarium solani*, *Fusarium oxysporum*, *Trichoderma reesei* and *Rhizopus oryzae*) were kindly provided by the Culture Collection and Identification Unit in the Regional Center for Mycology and Biotechnology, Al-Azhar University, Egypt. The fungal isolates were grown on Sabouraud Dextrose Agar (Difco, USA) plates. The plates were incubated for 5 days at room temperature. Sterile filter paper discs soaked with the purified enzyme were sited on agar plates. After incubation, the diameters of inhibition zones were measured.

N-acetyl-D-glucosamine (NAG) production by immobilized chitinase

Chitinous biowastes (prawn shells and fish shell wastes) were brought from local fish market, Benha, Egypt. Fishery wastes were washed twice with tap water, dried and rumpled in room temperature. The production of NAG by *Bacillus subtilis* was established by growing bacterial culture on the basal medium augmented with 0.2% various chitinous biowastes. The production medium was incubated at 37 °C with shaking at 250 rpm. The bacterial culture was then centrifuged at 12,000 rpm for 20 min. The absorbance was read at regular time periods (24-120 h). NAG concentration was determined in the supernatant using the standard curve of NAG. The production medium contained 0.2% colloidal

chitin was used as control in further work (Sashiwa et al., 2003; Kuk et al., 2005).

Statistical analysis

All data were expressed as the mean values of three replicates with the standard deviation (s.d.).

RESULTS AND DISCUSSION

Purification of chitinase from *Bacillus subtilis*

The crude chitinase produced from *Bacillus subtilis* was purified using 85% $(NH_4)_2SO_4$ saturation followed by dialysis, DEAE-Sepharose and Sephadex G-200 (Table 1).

The specific activity of chitinase was 52.1 Umg^{-1} protein with purification-fold and yield of 136-fold and 27 %, respectively. The molecular weight of the purified enzyme was about 52 KDa according to SDS-PAGE (Figure 2).

Mathur et al. (2011) recorded the production of chitinase from *Bacillus* sp. isolated from soil. Also, purification of chitinase from *Bacillus thuringiensis* was reported by Driss et al. (2005) and Kuzu et al. (2012). El-Shora et al. (2017) reported that the purification of chitinase through ammonium sulphate precipitation, DEAE-cellulose and Sephadex G-200 displayed specific activity of 51.9 Umg^{-1}protein and a molecular mass of 45 kDa.

Table 1: Purification profile of chitinase from *Bacillus subtilis*.

Purification schedule	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity Umg^{-1}	Purification fold	Yield (%)
Crude extract	480 ± 29	385 ± 2.6	1230 ± 6.3	3.2 ± 0.08	1.0 ± 0.02	100
Ammonium sulfate precipitation (85%)	144 ± 1.7	92.6 ± 1.5	1027 ± 5.4	11.1 ± 0.06	35 ± 0.7	84 ± 1.1
Dialysis	72 ± 1.2	40.4 ± 0.7	594 ± 3.2	14.7 ± 0.2	46 ± 0.9	48 ± 0.6
DEAE-Sepharose	36 ± 0.8	14.9 ± 0.4	439 ± 2.4	29.5 ± 0.4	93 ± 1.4	36 ± 0.8
Sephadex G-200	18 ± 0.3	6.3 ±0.2	328 ±0.15	52.1 ±0.9	163 ± 1.8	27 ± 0.02

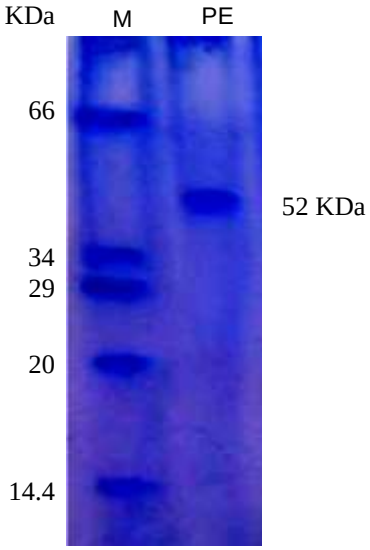

Figure 2: **SDS-PAGE of purified chitinase produced by *Bacillus subtilis*. Lane M: Markers and Lane PE: Purified enzyme.**

Effect of various compounds on purified chitinase

EGTA (Figure 3A) inhibited chitinase activity, indicating its role in the enzyme-catalytic activity. It was detected that thiol compounds (cysteine, DTT and CoA) enhanced chitinase activity (Figure 3B) indicating the essentiality of sulfhydryl group for enzyme catalysis. DTT enhanced the activity of other enzymes such as acid phosphatase from *Cladosporium cladospoioides* (El-Shora and Metwally, 2009). When purified enzyme was incubated with DTNB (Figure 3C) and TNM (Figure 3D), its activity was gradually inhibited. This revealed the essentiality of cysteinyl and tyrosyl groups for chitinase catalysis. Activity loss was detected in presence of DEPC (Figure 3E) indicating that the histidyl group is necessary for chitinase reaction. The necessity of arginyl residue for chitinase catalysis was affirmed by its inhibition by 2, 3-butanedione (Figure 3F). Similar results have been found by Zhikui et al. (2016) and Laribi-Habchi et al., (2014).

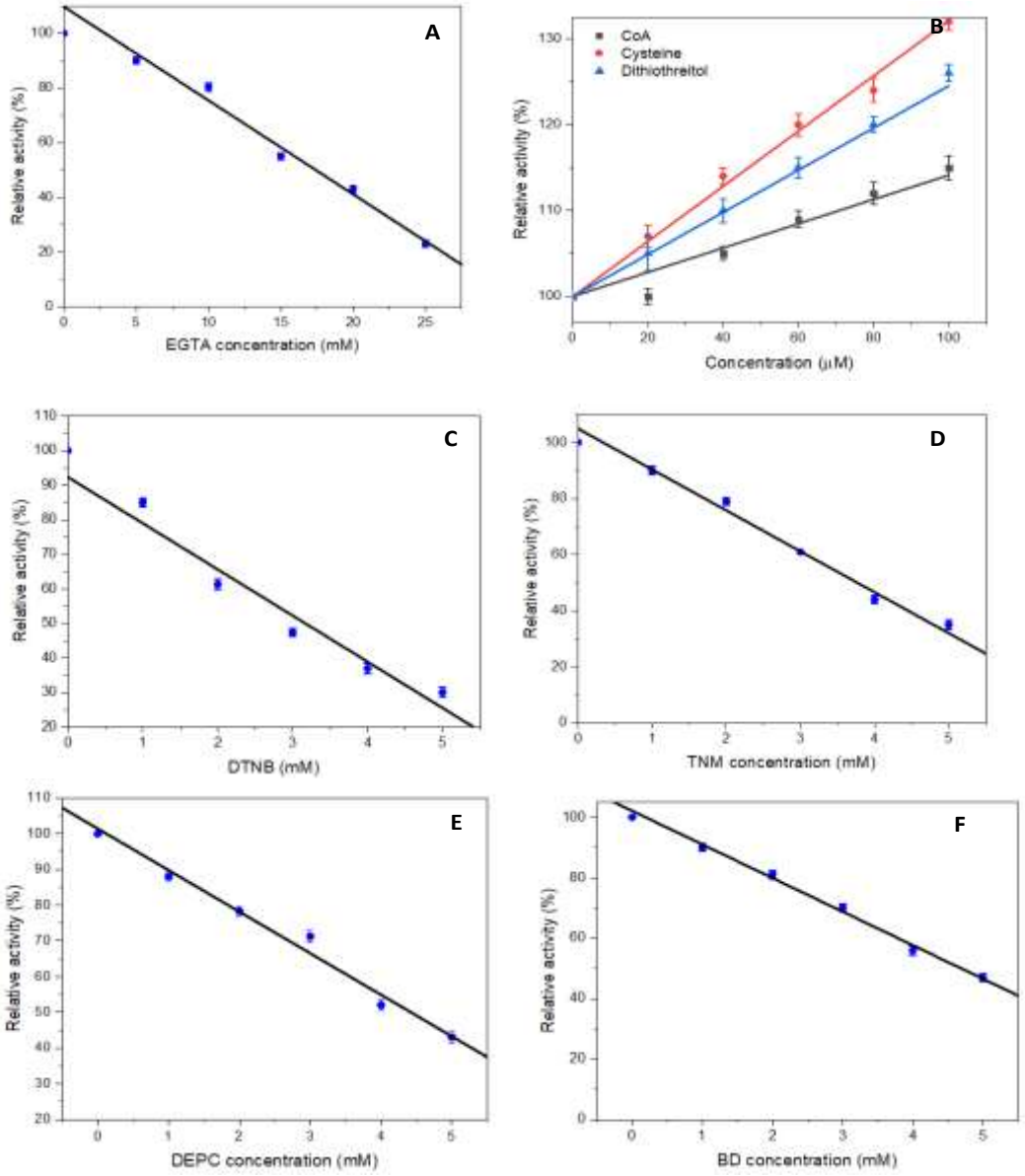

Figure 3: Effect of chemicals(A) EGTA, (B) thiol compounds, (C) DTNB, (D) TNM, (E) DEPC and (F) BD on purified chitinase obtained from *Bacillus subtilis*.

Immobilization of chitinase

The purified chitinase was immobilized on calcium alginate, silica gel and amberlite beads. The results depicted in Table 3 demonstrated the amount of immobilized enzyme on calcium alginate and was nearly 2-fold of that bounded to silica gel and 4-fold of that bounded to amberlite.

Table 2: Immobilization of the purified chitinase on different beads.

Beads	Added activity (U/mg protein)	Immobilized activity (U/mg protein)	% of Immobilization
Calcium alginate	87±1.7	59.8 ± 1.0	68.7 ± 0.8
Silica gel	87±1.2	33.9 ± 0.6	39.0 ± 0.7
Amberlite	87±1.5	15.4 ± 0.5	17.7 ± 0.4

Therefore, Ca-alginate beads were the best carrier for chitinase immobilization in the present work. El-Shora et al., (2017) reported that calcium alginate was better than silica gel for immobilization of chitinase. Prasad and Palanivelu (2013) described the successful immobilization of chitinase on chitosan beads. The results are in conformity with that obtained by Cheba et al. (2015) who propose that ionic binding of chitinase was the best method for immobilization process.

Effect of pH and temperature on the two forms of chitinase

The effect of different pH values on free and alginate-chitinase was measured using two different buffer systems with colloidal chitin as substrate (Figure 4).

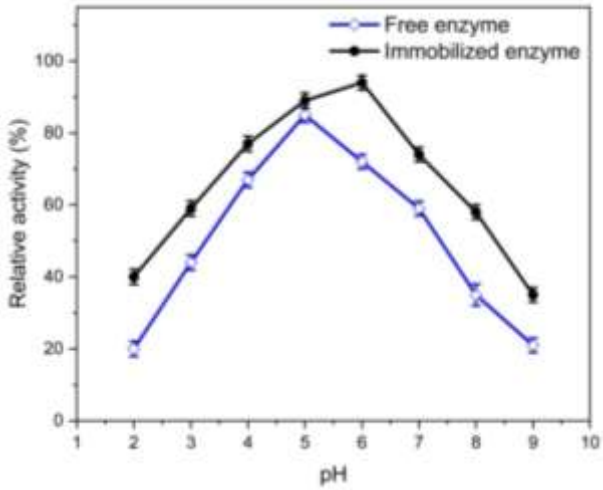

Figure 4: Effects of pH on free and immobilized chitinase from *Bacillus subtilis.*

The free and immobilized chitinase from *Bacillus subtilis* exhibited activity at broad pH range with the maximal activity at pH 5.0 and 6.0 for the two forms of chitinase, respectively. The shift of the optimal pH value of the immobilized chitinase to the alkaline region was possibly due to the change of its microenvironment initiated by the immobilization of the enzyme on the carrier. An increase of the optimal pH was observed for other immobilized enzymes (El-Shora et al., 2015; El-Shora and Youssef, 2008). The immobilized chitinase displayed higher stability over free enzyme in the pH range 4.0-7.0 (Figure 5 A,B). Considerable chitinase activity from *B. thuringiensis* was documented at pH range between 5.0 and 9.0 with a maximum value at pH 5.6 (Driss et al., 2005; Honda et al., 2017). Both free and immobilized chitinase from *B. thuringiensis* showed maximum activity at optimum pH value of 7.0 (Gomaa, 2012).

The temperature profile of free and immobilized chitinase when tested with colloidal chitin as substrate displayed the maximum activity at 50 ^{o}C and 60 ^{o}C, respectively (Figure 6A). Free chitinase from *Serratia marcescens* B4A (Zarei et al., 2011) exhibited optimal temperature at 45 ^{o}C. The shift in the optimum temperature of chitinase in the present work after immobilization process may be due to the protection of enzyme molecules during immobilization by formation of molecular cage around the enzyme molecules (Roger et al., 2004; Bai et al., 2006).

Thermostability of free and immobilized enzyme was carried out by pre-incubating enzyme for 80 min at 65 ^{o}C and 70 ^{o}C (Figure 6B). The free and immobilized chitinase reserved over 40% and 55% of its activity after 80 min at 60 ^{o}C, respectively. Meanwhile, the stability of free and immobilized chitinase at 70 ^{o}C displayed 37% and 27% of its original activity, respectively. Esawy et al. (2016) stated that the chitinase immobilization improved the sensitivity of the enzyme toward temperature compared to its free form. It was reported that the thermostability of free and immobilized chitinase at 60^{o}C was better than that at 70 ^{o}C (El-Shora et al., 2017).

Effect of metal ions on free and immobilized chitinase

The free and immobilized chitinase exhibited a positive stimulatory effect by Co^{2+} and Mn^{2+}. Chitinase activity was moderately inhibited by Fe^{3+}, Cd^{2+}. Hg^{2+} was the most potent inhibitor. $Ca^{2+}$and Mg^{2+} did not show any remarkable effect on chitinase (Table 3).

Table 3: Effect of metal ions on free and immobilized chitinase activity.

Metal ions (5 mM)	Relative activity (%)	
	Free chitinase	Immobilized chitinase
None	100	100
Mg^{2+}	100 ± 2.1	101 ± 1.6
Mn^{2+}	114 ± 1.2	125 ±1.3
Fe^{3+}	27 ± 5.3	31 ± 0.7
Hg^{2+}	1.2 ± 0.02	1.9 ± 0.03
Ca^{2+}	97 ± 1.7	98 ± 1.6
Cu^{2+}	43 ± 1.8	61 ± 1.7
Co^{2+}	118 ± 0.8	131 ± 0.9
Cd^{2+}	12 ± 0.6	14 ± 0.3

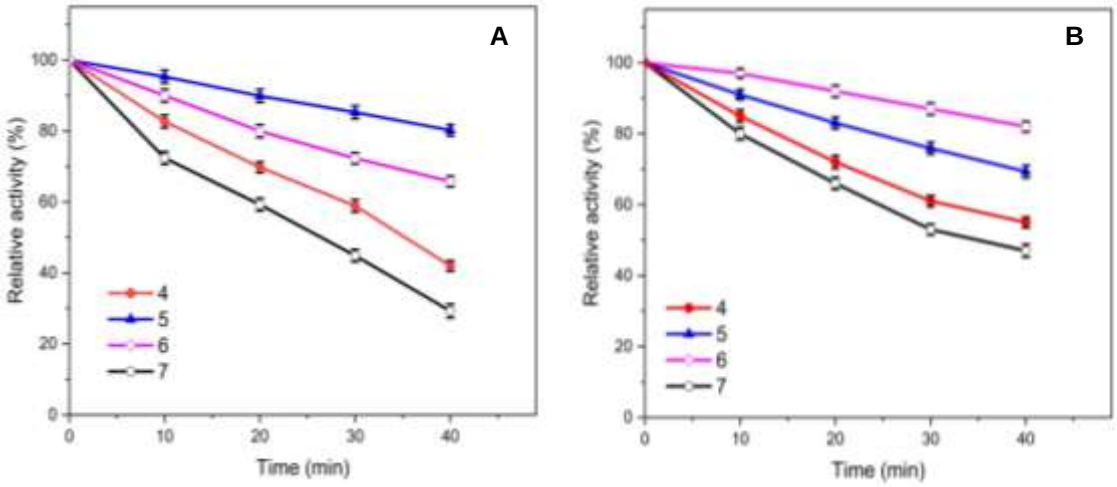

Figure 5: pH stability of free (A) and immobilized chitinase (B) from *Bacillus subtilis.*

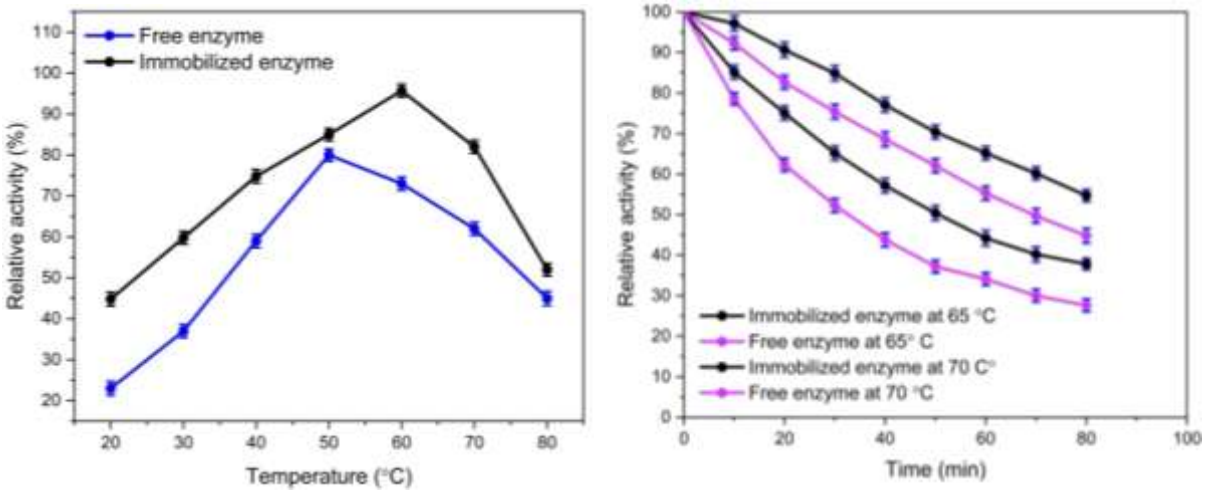

Figure 6: (A) Effect of the temperature on free and immobilized chitinase from *Bacillus subtilis*, (B) Thermal stability of free and immobilized chitinase at 65 ºC and 70 ºC.

Honda et al. (2017) stated that chitinase from *B. thuringiensis* is a metalloenzyme activated by Ca^{2+}, Mg^{2+} and Cu^{2+} and inhibited by some metal ions such as Cd^{2+} and Hg^{2+}. On contrary, chitinase activity was inhibited by other metals such as Ag^{+}, Cd^{2+}, Hg^{2+} and Pb^{2+} (Dahiya et al., 2005; Woo-Jin et al., 2005).

Kinetic properties of free and immobilized chitinase

Each of the two forms of chitinase followed Michaelis-Menten kinetics. The results depicted in Figure 7 showed a Lineweaver-Bürk plot and the kinetic constants, K_m and V_{max}, for free and immobilized chitinase were calculated. The K_m values were 1.7 and 2.7 $mgml^{-1}$ whereas V_{max} values were143 and 294 Umg^{-1} protein for free and immobilized alginate-chitinase, respectively. The K_m values indicated that the free chitinase is more responsive to the substrate compared to immobilized enzyme. In addition, the V_{max} values revealed that the immobilized enzyme is faster than free form. Prasad and Palanivelu (2013) reported that the K_m values for free and immobilized chitinase were 0.7 and 1.3 $mgml^{-1}$, respectively. The values of V_{max} for free and alginate-immobilized chitinase were 4.5 and 82.5 Umg^{-1} protein, respectively. These results are also in harmony with those of Esawy et al. (2016) who found higher V_{max} (40.0 Umg^{-1} protein) for immobilized enzyme than the free one (13.33 Umg^{-1} protein).

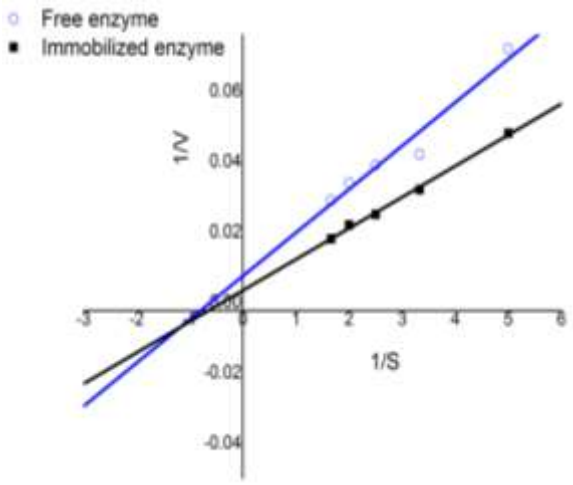

Figure 7: Lineweaver-Bürk plot of the free and immobilized chitinase with colloidal chitin as substrate.

Antifungal activity of purified chitinase

The antifungal activity of the free chitinase from *Bacillus subtilis* was examined against *Aspergillus oryzae*, *Rhizoctonia solani*, *Fusarium solani*, *Fusarium oxysporum*, *Trichoderma reesei* and *Rhizopus oryzae* (Figure 8).

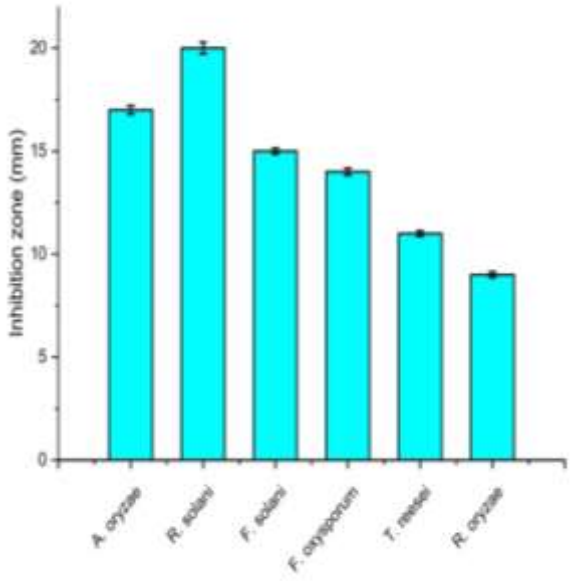

Figure 8: Antifungal activity of the purified chitinase from *Bacillus subtilis.*

The results revealed that chitinase expressed a powerful antifungal activity against all the tested fungi, particularly *Rhizoctonia solani*. However, minimum inhibition zone was recorded for *Rhizopus oryzae*. In support, the antifungal activity of chitinase was observed by other researchers (Kuddus, 2014; Stoykov et al., 2015). Chitinase from *Serratia marcescens* B4A and *A. niveus* exhibited antifungal activity against several fungi (Alves et al., 2018;Kurniawan et al., 2019). The biocontrol activity by *Bacillus* chitinase was investigated by many researchers (Saber et al., 2015; Iqbal and Anwar, 2019; Kurniawan et al., 2019).

Production of NAG by immobilized chitinase

Two fishery wastes were tested for the production of NAG using immobilized chitinase from *Bacillus subtilis* comparing to colloidal chitin. The best substrate for the production of NAG was colloidal chitin (Figure 9).

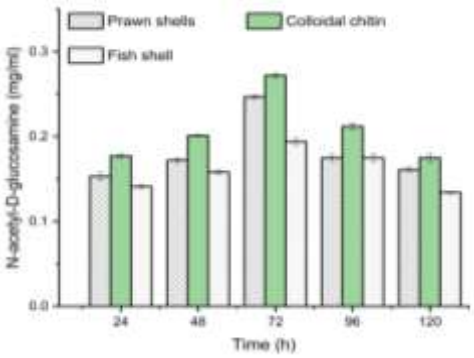

Figure 9: Production of N-acetyl-D-glucosamine from fishery wastes using the immobilized chitinase from *Bacillus subtilis*

Higher production of NAG by immobilized chitinase was recorded during biodegradation of prawn shells waste compared to shrimp shell wastes. The maximum production of NAG was observed after 72 h of incubation for chitinous wastes. The results are similar to those found by Das et al. (2019) who reported production of NAG by chitinase from marine fungi through biodegradation of chitins wastes.

CONCLUSION

Chitinase from *Bacillus subtilis* was purified to homogeneity using 85% $(NH_4)_2SO_4$ followed by dialysis, DEAE-Sepharose and Sephadex G-200. Among the adopted immobilization strategies, Ca-alginate displayed a promising immobilization yield. The immobilized biocatalyst showed prominent improvement in stability toward temperature and pH. The purified chitinase showed a powerful biocontrol activity against numerous fungi. Prawn shells waste was the best substrate for NAG production by immobilized chitinase comparing to shrimp shell wastes.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

El-Shora and El-Sharkawy designed and performed the experiments together. They calculated the data, wrote and reviewed the manuscript. In addition, the two authors read and approved the final version.

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