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Single step hydrolysis of chitin using thermophilic immobilized exochitinase on carrageenan-guar gum gel beads

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ABSTRACT

Chitinases are chitin glycosyl hydrolases that can be employed in various biotechnological applications. In the present study, exochitinase produced by *Alternaria* sp. strain Sha was immobilized on carrageenan-guar gum gel beads with immobilization yield of 43% improved to reach 100% by statistical optimization using central composite design. The coefficient of determination value for the applied model was 0.903 and the absolute average deviation was 8.9% confirming that the applied model was successfully used in the optimization process. Fourier Transform Infrared Spectroscopy, Thermal Gravimetric analysis and Scanning Electron Microscopy was used to examine the stepwise different formulations of the beads as well as the immobilized one, confirming the success of the immobilization process. The immobilized enzyme activity was optimized in respect to the effect of temperature, pH and substrate concentration indicating that the immobilized enzyme was optimally active at 60°C and pH 5 with suitable activation energy (20.287 ± 0.159kJmol^{-1}), K_m and V_{max} values of 2 mg/mL and 5.9U/mg protein/min respectively. Moreover, the immobilized enzyme showed high thermal and operational stabilities, it retained more than 90% of its activity after 2 h at 65°C and it retained 100% of its activity for more than 15 cycles as well as it kept its complete activity at 4°C for more than one month. Finally, the immobilized enzyme was successfully employed for the direct hydrolysis of crab chitin and production of chitooligosaccharides as it produced reducing sugars of 193.8 μg/mg chitin after 12 h, confirmed by thin layer chromatography analysis and Fourier Transform Infrared Spectroscopy.

1. Introduction

Chitin is a linear β-1,4-glycosidic linkage chain of N-acetyl-D-glucosamine that considered to be the most abundant biopolymer exist in nature other than cellulose. It presents in fungi, yeasts, insects, crustacean shells, and other invertebrates (Young et al., 2005). Chitin hydrolysis products as chitooligosaccharides (COS), N-acetyl-D-glucosamine (GlcNAc) and glucosamine (GlcN) have attracted a growing interest due to their various health benefits (Das et al., 2019, Liang et al., 2018, Liaqat and Eltem, 2018 and Zhang and Yan, 2017).

The hydrolysis of chitin can be achieved by either chemical or enzymatic mediated processes. The enzymatic mediated processes are advantageous as they overcome the low yield, low product quality and the environmental pollution hazards of the chemical mediated processes (Das et al., 2019 and Zhang et al., 2018). However, the high crystalline structure of chitin resulted from the hydrogen bond between the chains hinders the enzymatic hydrolysis of chitin. Several studies have been investigated for increasing solubility and/or decreasing crystallinity of chitin in order to improve enzymes accessibility and consequently improve the hydrolytic activity. Acidification, bacterial fermentation, ultrasonication, high-pressure homogenization and the use of ionic liquids are some examples of the investigated methods that showed a positive value in improving the enzymatic hydrolysis of chitin (Chen et al., 2015; Husson et al., 2017; Villa-Lerma et al., 2013; Wei et al., 2017; Zhang et al., 2018). However, large-scale processing using these suggested methods are difficult due to the high cost, environmental pollution or equipment limitations reflecting the importance for achieving direct hydrolysis of chitin to overcome these limitations.

Chitinases are chitin glycosyl hydrolases, classified into endochitinases that act randomly within the chain and exochitinase that hydrolyze the chain from the terminal end leading to the production of diacetylchitobiose or N-acetylglucosamine units (Sahai and Manocha,

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1993). They gained a great attention due to their various biological and biotechnological applications in food, agricultural and medical industry as well as their use in the biorefinery of crustaceans byproducts (Zhang et al., 2018; Halder et al., 2019).

COS are the water soluble partial hydrolysis products of chitin or its deacetylated derivative (chitosan) that composed of 2–20 units of either GlcN and/or GlcNAc with an average molecular weight < 3900Da. They posses various biological activities as antitumor, anti-inflammatory and immuno-modulatory effect reported in our previous researches (Hashem et al., 2018; Mohamed et al., 2018) in addition to other activities as antioxidant, antimicrobial, anti-hypertensive, hypocholesterimic and neuroprotective effects (Liang et al., 2018; Liaqat and Eltem, 2018; Sanchez et al., 2017; Zou et al., 2016). The industrial application of chitinases in the hydrolysis of chitin and production of COS is limited due to their high cost so immobilization of the enzyme is necessary. Immobilization not only offers the reusability of the enzyme which decrease the total cost of the enzyme but also it will stabilize the enzyme structure allowing its application even in drastic environmental conditions of temperature, pH and organic solvents (Prasad and Palanivelu, 2014; Esawy et al., 2016).

Several techniques can be used for the immobilization of the enzyme, such as adsorption, entrapment, encapsulation, cross linking and covalent binding (Salman et al., 2008). Covalent binding offers the advantage of avoiding the enzyme leakage from the carrier, make this technique more preferred in industrial scale (Danial et al., 2010). Several biopolymers as alginate, carrageenan and chitosan have been previously used for the immobilization of chitinases (Seo et al., 2012, Esawy et al., 2016 and El-Shora et al., 2018). The current study focused on the use of carrageenan in addition to guar gum for the preparation of micro carrier used in the covalent binding immobilization of *Alternaria* sp. strain Sha exochitinase followed by the optimization of the immobilization process using statistical technique. In addition, the optimum conditions for the activity of the immobilized enzyme and its kinetic constants were studied. Finally, the activity of the immobilized enzyme in the direct hydrolysis of crab chitin and the production of COS were examined.

2. Materials and methods

2.1. Materials

Wheat bran and sugarcane bagasse were purchased from the local market. Crab chitin, 4-NitrophenylN-acetyl-β-D-glucosaminide, 4-Nitrophenol, N-acetyl glucosamine, glucosamine, carrageenan and guar gum were purchased from Sigma-Aldrich, Saint Louis, USA. Chitopentose and chitohexose were purchased from Seikagaku Biobusiness Corporation, Tokyo, Japan. Dinitrosalicylic acid (DNS) was obtained from Panreac, Barcelona, Spain. Potato dextrose agar (PDA) medium and silica gel 60 thin-layer chromatography (TLC) plates were purchased from Merck, Darmstadt, Germany. All other chemicals were of analytical or HPLC grade.

2.2. Production of chitinase

The fungus *Alternaria* sp. strain Sha (Genbank accession no.**MK139827**) was used for the production of chitinase using solid state fermentation as previously described by Ismail et al. (2019). Briefly, 250 mL Erlenmeyer flask containing (g/flask) wheat bran; 5, sugarcane bagasse; 1.5 and chitin; 0.075 moistened with 5 mL of salt solution (composed of (g%) $MgSO_4$; 0.012, KH_2PO_4; 0.015, $NH_4H_2PO_4$; 0.05, peptone; 0.02 and yeast extract; 0.45, pH 5), inoculated with 2 mL of spore suspension prepared from 5 days old age fungus (on potato dextrose agar) then incubated at 25 °C for 16 days. Extraction of the enzyme was carried out by adding 50 mL of distilled water, mixing in a rotary shaker at 150 rpm for 1 h followed by centrifugation at 4 °C for 10 min at 5000 rpm.

2.3. Enzyme assay and protein content

The enzyme activity was determined by the hydrolysis of 0.1% of the synthetic substrate 4-NitrophenylN-acetyl-β-D-glucosaminide in 0.05 M acetate buffer pH 4.5 (Rustiguel et al., 2012). The released p-nitrophenol upon ionization in the alkaline pH formed the yellow p-nitrophenylate ion. The absorbance of the p-nitrophenylate ion was measured at 410 nm. One unitof the enzyme was defined as the amount of enzyme that released 1 µmol of p-Nitro-phenol (equivalent to 1 µmol of N-acetylglucosamine) per minute under the assay conditions.

The protein content was determined using Lowry-Folin method against a bovine serum albumin standard (Lowry et al., 1951).

2.4. Gel beads preparation and activation

Carrageenan guar gum gel beads were prepared by dissolving carrageenan in distilled water at 70 °C. After complete dissolution; the guar gum solution with final concentration 2% of the two polymers (the ratio between carrageenan and guar gum is 2:1) was added. It was left in hot water bath till the air babbles disappeared and the mixture become homogenous then the solution was dropped by using syringe needle in hardening solution of 2% KCl. After 3 h remaining in hardening solution, the generated gel beads then soaked in 4% polyethyleneimine (PEI) solution for 3 h. The un-reacted PEI was removed by successive washing using distilled water. The aminated beads were soaked in 2.5% of glutaraldehyde solution (GA). After 3 h, the gel beads were washed well with distilled water to remove un-reacted GA then the gel beads became active and ready for immobilization step.

2.5. Immobilization

The produced enzyme was immobilized on carrageenan-guar gum gel beads in which 1 g of the beads was soaked for 6 h at room temperature with the enzyme solution of 10.6U unless otherwise is described. After immobilization, the beads were washed twice with distilled water to remove any unbound enzyme. The washed beads were immersed in distilled water and were stored at 4°C for further measurements. The immobilization yield was calculated as follow:

$$\text{Immobilization yield (\%)} = I / (A\text{-}B) \quad (1)$$

A is the enzyme added (U/carrier), B is the unboundenzyme (U/carrier) and I is the immobilized enzyme (U/carrier).

The amount of protein immobilized on the gel carrier Pg (mg/g) was calculated using the following equation:

$$Pg = C_oV_o - C_fV_f / W \quad (2)$$

where C_o is the initial protein concentration (mg/mL), C_f the protein concentration of the filtrate (mg/mL), V_o the initial volume of the enzyme solution (mL), V_f the volume of filtrate (mL), and w is the weight of gel carrier used (g).

2.6. Statistical optimization of the immobilization process

Central composite design (CCD) is an experimental design developed for the first time by Box and Wilson (1951). In the current study, CCD with four-star points and five-replicates at the center point was applied. The independent variables, loading time (X_1) and enzyme units added (X_2) were examined in 13 experimental runs. The second order polynomial function to correlate relationship between independent variables and response of immobilized yield was as follow:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_1\beta_2X_1X_2 \quad (3)$$

where Y is the immobilized yield (%), β_0 is the intercept, β_1 and β_2 are linear coefficients, β_{11} and β_{22} are quadratic coefficients, β_{12} is cross product coefficients.

2.7. Fourier transform infrared (FT-IR)

The infrared spectra of all formulations (Carrageenan-guar gum), (Carrageenan-guar gum + PEI), (Carrageenan-guar gum + PEI + GA) and (Carrageenan-guar gum + PEI + GA + Enzyme) were recorded with Fourier transform infrared spectroscopy (FTIR-8300, Shimadzu, Japan).

2.8. Thermal Gravimetric analysis (TGA)

TGA was performed to prove the formation of a strong poly electrolyte complex. The thermal behavior of the different gel formulations was characterized by the TGA (PERKIN ELMER TGA7).

2.9. Scanning electron microscope (SEM)

Surface of different gel formulations were examined by field emission high resolution scanning electron microscope (quanta 250).

2.10. Effect of temperature and pH

The effect of pH on the enzyme activity was evaluated using 0.05 M acetate buffer in pH range from 3.5 to 5.5. The pH at which the enzyme processed the highest activity was considered as optimum. In addition, the stability of the enzyme at the considered pH range was determined by estimating the residual activity of the enzyme every 30min up to 2 h after its pre-incubation at each pH individually. The activity of the enzyme without pre-incubation was considered as 100% activity.

The effect of the temperature on the activity of the immobilized enzyme was determined at various temperatures (45-65°C) at the optimum pH. The activation energy (E_a) for the produced enzyme was calculated from Arrhenius plot (ln relative activity versus reciprocal of temperature in Kelvin), as given in the following equation:

$$\text{Slope} = -E_a/\text{R} \quad (4)$$

in which R is the gas constant.

2.11. Effect of substrate concentration and kinetic constants determination

The effect of different substrate concentrations (0.1–4 mg/ml) on the activity of the immobilized enzyme was studied at the optimum conditions. The kinetic constants were calculated on the base of Lineweaver-Burk plot (Lineweaver and Burk, 1934) by plotting of the reciprocal of the specific activity (U/mg protein) versus the reciprocal of the substrate concentration (g/mL).

2.12. Thermal stability

Thermal stability of the immobilized enzyme was determined by estimating the residual activity of the enzyme at the optimum conditions every 30min up to 2 h after its pre-incubation at temperature range from 45 to 65°C. The activity of the enzyme without pre-incubation was considered as 100% activity.

2.13. Enzyme reusability

The ability of the immobilized enzyme to hydrolyze 4-NitrophenylN-acetyl-β-D-glucosaminide for several times was examined at the optimum conditions. At the end of the reaction, the gel beads were removed, washed and used in a new reaction. The amount of the released p-nitrophenol in the supernatant after each reaction was determined as previously mentioned. In order to determine the storage stability of the immobilized enzyme, the gel beads were immersed in distilled water and stored at 4°C then the activity of the enzyme was measured every week for 1 month.

2.14. Hydrolysis of chitin

2.14.1. Enzymatic hydrolysis using the immobilized enzyme

The activity of the immobilized enzyme in the hydrolysis of crab chitin was examined in a reaction mixture of 1 g bead (2.65U) immersed in 1 mL distilled water and 0.5 mL of 1% chitin in 0.05 M acetate buffer pH 5 incubated at 30°C, 150 rpm for different hydrolysis periods. At the end of the hydrolysis period, the beads were removed from the solution then the un-hydrolyzed portion of the substrate in the solution was removed by centrifugation at 4 °C for 10 min at 10,000 rpm. The amount of the released reducing sugars in the supernatant was determined by DNS method (Miller, 1959).

2.14.2. Acid hydrolysis

Acid hydrolysis was carried out using concentrated HCl in which 50 mL of the acid was added to 1 g of crab chitin then incubated at 30°C for 30min. The reaction was stopped by its immersion in ice bath. The HCl was evaporated under vacuum and the residual was suspended in distilled water. Finally the solution bought to neutral pH by using 10 M NaOH (Cabrera and Cutsem, 2005).

2.14.3. TLC analysis of the chitin hydrolyzed product

The chitin hydrolyzed products were identified by TLC using a mixture of propanol: water: ammonia (7: 2: 1 v/v) as a mobile phase (Cabrera and Cutsem, 2005). The resulted sugars were visualized with diphenyl amine-aniline reagent (Tanaka et al., 1999).

2.14.4. Chemistry of the chitin hydrolyzed product

The functional groups and chemical bonds of the final hydrolysis product of chitin using the immobilized enzyme were determined through FTIR analysis.

2.15. Statistical analysis

All experiments were performed in triplicates with three measurements per replicate and the averages of the results were reported.

3. Results and discussion

3.1. Enzyme activity

The fungus *Alternaria* sp. strain Sha under the fermentation condition described in the above section produce chitinolytic enzyme of 2.65U/mL for exo-activity with total protein content of 2.14 mg/mL. Accordingly, the calculated specific activity was 1.24U/mg protein. As previously reported (Ismail et al., 2019), this strain of *Alternaria* sp. produces high amount of exochitinase in the presence of chitin as an inducer. The production of chitinases was very expressive that explained by their catalytic mechanisms in the hydrolysis of chitin (Das et al., 2019 and Halder et al., 2019). The reusability of any enzyme is an important parameter in the determination of the economic viability of its industrial use (Fernandes et al., 2013), reflecting the importance of the immobilization of the produced enzyme.

3.2. Immobilization conditions of the produced enzyme on carrageenan-guar gum gel beads

The produced enzyme was immobilized by covalent binding on carrageenan-guar gum gel beads with almost 43% immobilization yield. The immobilization process was optimized by applying CCD. The results in Table 1 indicated that almost 100% immobilization yield was achieved by loading of 2.65U of the enzyme to 1 g of the gel beads for 6 h. The specific activity calculated on the bound-protein basis was 1.76U/mg protein with 1.42 fold of purification. The resulted immobilization yield was similar to that reported by Esawy et al. (2016) but it was higher than 47.4% reported by Fernandes et al. (2013),

Table 1
CCD for optimization of the immobilization process.

Trial	Independent variable		Observed immobilization yield (%)	Predicted immobilization yield (%)	Residual
	X_1 Time of loading (h)	X_2 Loading unit (U/g gel bead)			
1	3 (-1)	5.3 (-1)	48.83	64.07	−15.24
2	3 (-1)	15.9 (+1)	29.24	22.53	6.71
3	18 (+1)	5.3 (-1)	72.64	77.81	−5.18
4	18 (+1)	15.9 (+1)	29.20	28.89	0.31
5	0.5 (-∞)	10.6 (0)	34.18	33.03	1.15
6	25 (+∞)	10.6 (0)	43.36	41.30	2.06
7	6 (0)	2.65 (-∞)	99.96	86.95	13.01
8	6 (0)	21.2 (+∞)	17.11	21.07	−3.97
9	6 (0)	10.6 (0)	41.91	42.60	−0.69
10	6 (0)	10.6 (0)	41.91	42.60	−0.69
11	6 (0)	10.6 (0)	44.01	42.60	1.41
12	6 (0)	10.6 (0)	42.95	42.60	0.35
13	6 (0)	10.6 (0)	43.36	42.60	0.76

72.9% reported by Cheba et al. (2015), 88% reported by El-Shora et al. (2018) and 96.12% reported by Preety and Hooda (2018).

The multiple regression analysis of the data (Table 2) indicated that both of the examined variables (loading unit and time of loading) significantly affected the immobilization yield ($P < 0.05$). Also it indicated the accuracy of the applied model since the R^2 value of the model was 0.903, reflecting that the second order polynomial model can explain 90.3% of the variation in the experimental results. Edwards et al. (2008) reported that the R^2 value (> 0.9) of the applied model indicates its accuracy. However, Yolmeh and Jafari (2017) reported that the accuracy of the model cannot be demonstrated by the R^2 index alone and the absolute average deviation (AAD) is a better measure of the accuracy. Based on that, the accuracy of the model was confirmed by the low value of AAD (8.9) calculated by the following equation:

$$AAD = \left\{ \left[\sum_{i=1}^{p} (|Yexp - Yprd|/Yexp) \right] /P \right\} X\ 100 \quad (5)$$

where P, Yexp and Yprd are the number of the experiment, observed and predicted immobilization yield, respectively. The suitable values of R^2 and AAD confirmed that the applied model depicts the correct behavior and it can be successfully used in the optimization process (Ghorbannezhad et al., 2016).

The analysis of variance (ANOVA) has been calculated and it indicated that the model terms used in that research are statistically significant, as an evident from the high F value (61.59) and the very low P value (8.98E-16). The second order polynomial equation, concluded from the regression analysis of the experimental results, used for

Table 2
Analysis of CCD.

Term	Regression coefficient	Standard error	t- test	P-value
Intercept	93.492	7.528	12.42	5.49E-14
X_1	2.712	0.87	3.119	0.003754
X_2	−7.835	1.002	−7.82	5.18E-09
X_1^2	−0.074	0.028	−2.682	0.011336
X_2^2	0.191	0.037	5.130	1.26E-05
X_1X_2	−0.046	0.045	−1.034	0.308484
Model summary				
Multiple R				0.950
R^2				0.903
Adjusted R^2				0.889
Standard Error				6.782

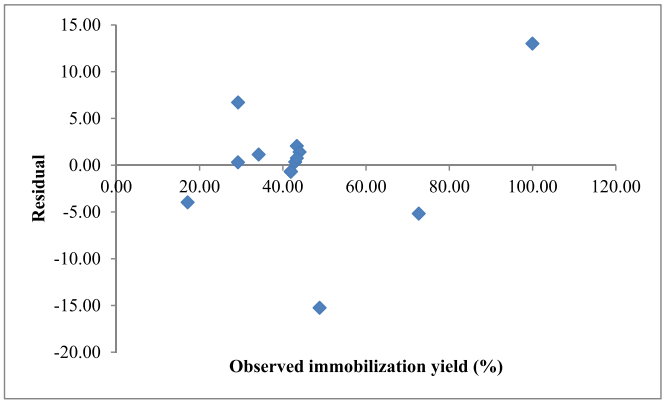

Fig. 1. Residual plot.

the calculation of the predicted immobilization yield was:

$$Y_{\text{Immobilization yield}} = 93.4923 + 2.712X_1 - 7.83481X_2 - 0.0738 \times {}_2^2 + 0.191285 \times {}_2^2 - 0.04646 \times 1 \times {}_2 \quad (7)$$

Moreover, the residual analysis (Fig. 1) obtained by plotting of the observed – predicted values (residuals) versus the response (immobilization yield), indicated that the residuals were fallen in a symmetrical pattern and were constantly spread throughout the range, ensuring that the model is correct on average for all observed results.

3.3. Fourier transform infrared

FT-IR characterizations of all formula (A-D) were shown in figure (2). For the first formula (A); there are two peaks at 620 cm^{-1} and 1080 cm^{-1} that corresponded to (-OSO3-) group of carrageenan. Also the peaks at 1632 cm^{-1} were attributed to carboxylic groups of the carrageenan (–COO–) and at 3453 cm^{-1} were corresponding to (-OH) groups of guar gum. While in formula (B); there are two characteristic peaks attributed to amine groups of PEI found on the surface of gel beads. The first one was at 1634 cm^{-1} that represent the (–COO–) groups and the other one at 3438 cm^{-1}that of the amine groups that overlapped with peak of hydroxyl groups found on the gel beads. In formula (C), that contains activated gel beads with GA, the peaks at 3436 cm^{-1} is corresponding to the hydroxyl groups of GA is overlapped with that of amine groups and also hydroxyl groups that originally found on the gel beads surface. This formula also has the main characteristic peak at 1634 cm^{-1} that is corresponding to (-C═N-) pond that is formed during Schiff base interaction between amine groups found on the gel beads after amination step and the aldehyde groups of the GA. Finally for formula (D); it has a peak at 3436 cm^{-1} that corresponding to the amine groups of the enzyme that overlapped with the peak of amine groups and hydroxyl groups found already on the gel beads surface, also it contains peak at 1634 cm^{-1} that related to (-C═N-) pond which formed during Schiff base interaction between amine groups of the enzyme and the aldehyde groups of the GA that found on the gel beads surface. This result agrees with that reported by Wahba and Hassan (2017).

3.4. Thermal Gravimetric analysis

Treatment of carrageenan-guar gum and its modification by PEI and GA has shown gradual and obvious improvement in the TGA and DSC of the gel beads. The TGA of the carrageenan-guar gum and the modified formulas (A-D) showed better stability against thermal degradation as shown in Table 3 and figure 3A. In this figure, carrageenan-guar gum had degradation at 100 and 200 °C. These values have been increased to become 150 and 250 °C after amination process using PEI. Also, it increased to 200, 300 and 700 °C after activation with GA and finally

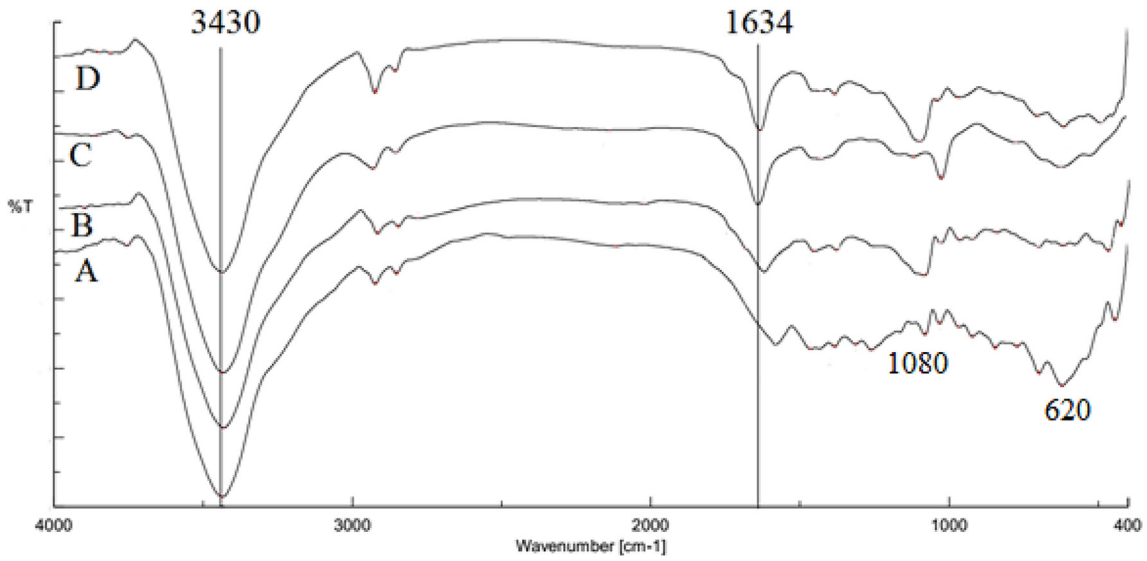

Fig. 2. FT-IR spectra of (A) Carrageenan-guar gum, (B) Carrageenan-guar gum + PEI, (C) Carrageenan-guar gum + PEI + GA and (D) Carrageenan-guar gum + PEI + GA + Enzyme.

Table 3
The main values of TGA and DSC thermogarms

Sample	DSC	TGA	T_{50}
(A) Carrageenan-guar gum	211	100, 200	383
(B) Carrageenan-guar gum + PEI	224	150, 250	577
(C) Carrageenan-guar gum + PEI + GA	255, 320, 436	200, 300, 700	736
(D) Carrageenan-guar gum + PEI + GA + Enzyme	246, 309, 895	230, 350, 850	1000

became 230, 350 and 850 °C after immobilization. The gel beads thermal improvement could be explained by the formation of polyelectrolyte interaction between sulfonic groups (polyanion) found in the carrageenan and the amine groups (polycation) found in PEI. Further interaction with GA showed much higher increase in the thermal stability of the gel beads and the degradation behavior became slower and gradual. This improvement in TGA could be attributed to the interaction between amine groups of the PEI on the surface of the gel beads and the carbonyl group of the GA forming strong amide bond. When we calculated the T_{50}, the temperature at which 50% of the sample has been decomposed, we found that it increased gradually with further treatment from 383 °C to about 1000 °C, as in Table 1.

On the other hand, the DSC exothermic effect has been shifted to much higher temperatures as shown in Table 3 and figure 3B. The carrageenan-guar gum has shown exothermic band at 211 °C, which has

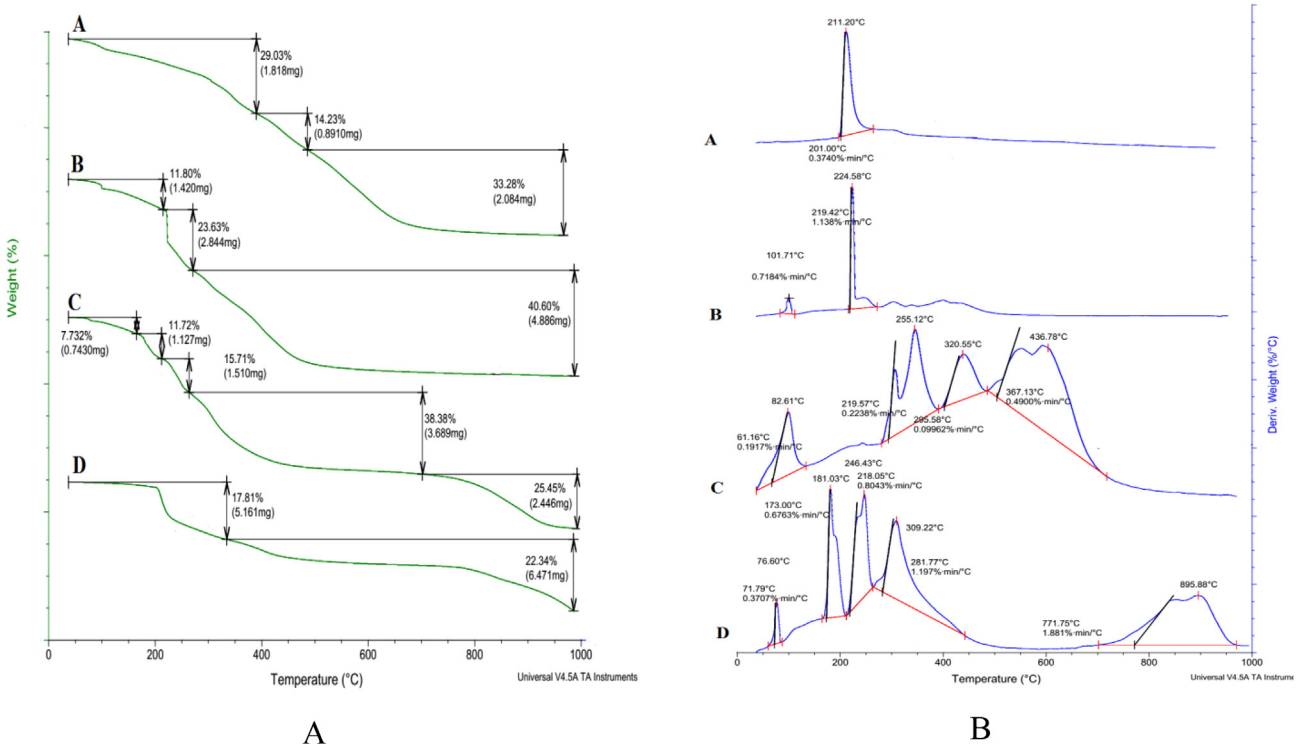

Fig. 3. TGA (A) and DSC (B) thermogram.

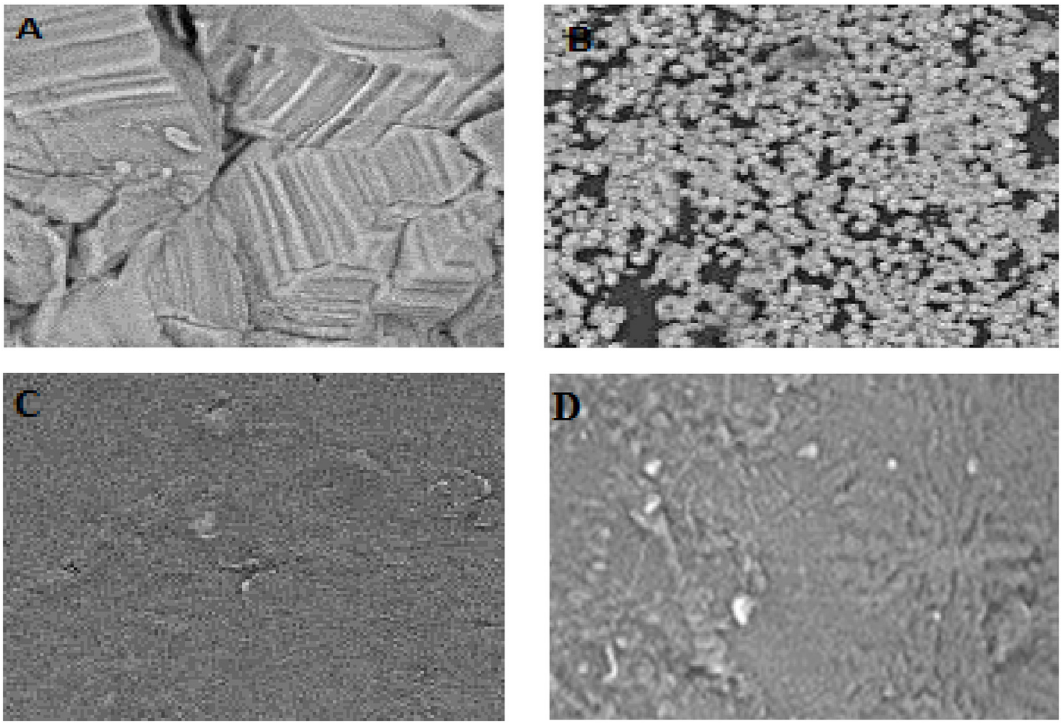

Fig. 4. SEM of (A) Carrageenan-guar gum, (B) Carrageenan-guar gum + PEI, (C) Carrageenan-guar gum + PEI + GA and (D) Carrageenan-guar gum + PEI + GA + Enzyme.

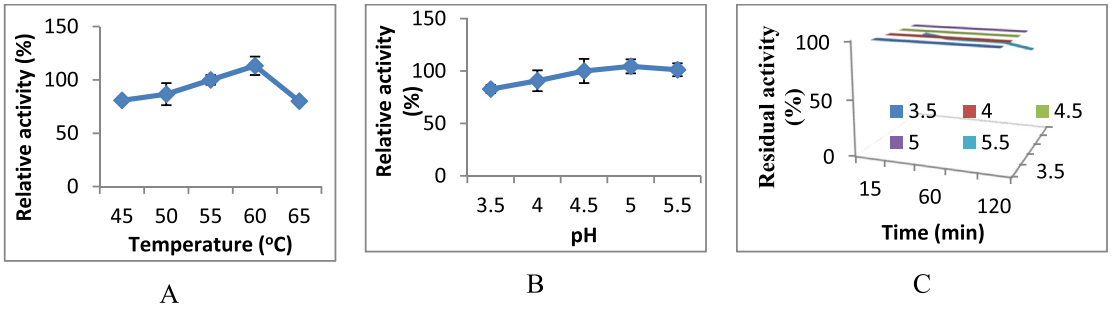

Fig. 5. Effect of (A) the reaction temperature (55°C is the control) (B) the reaction pH (control is pH 4.5) (C) pre-incubation at different pHs for different time intervals (the activity of the enzyme without pre-incubation was considered as 100% activity) on the activity of the immobilized enzyme.

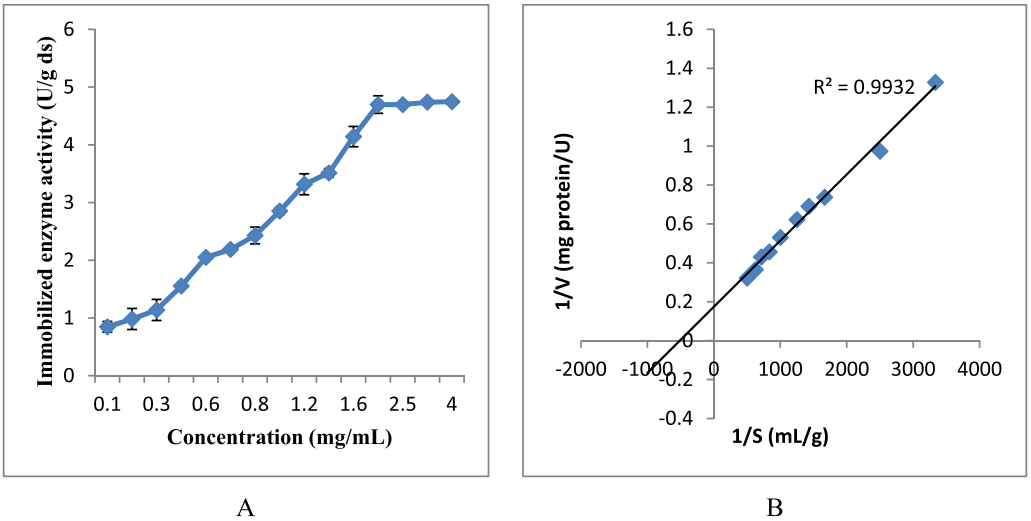

Fig. 6. The activity of the immobilized enzyme using different substrate concentrations (A) and Lineweaver-Burk plot (B).

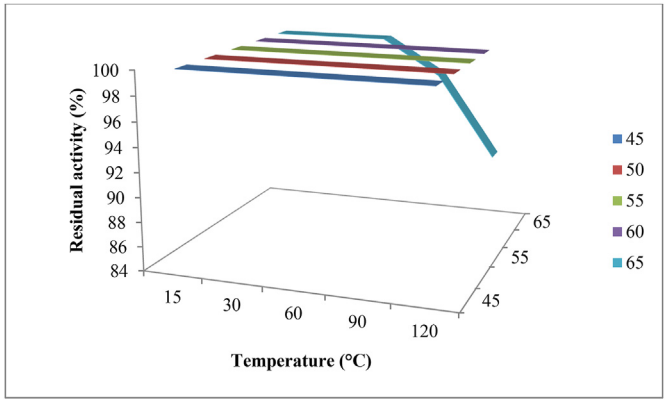

Fig. 7. Thermal stability (the activity of the enzyme without pre-incubation was considered as 100% activity).

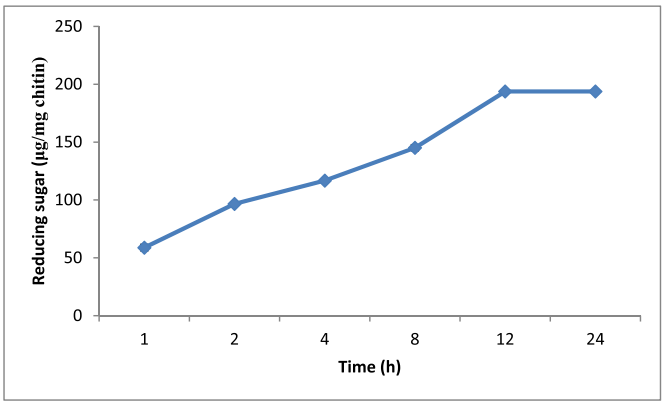

Fig. 8. The amount of the released reducing sugar by the hydrolysis of chitin using the immobilized enzyme.

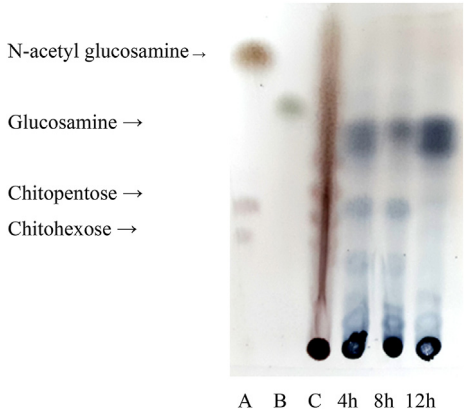

Fig. 9. TLC plate of chitooligosaccharides produced by the direct hydrolysis of crab chitin using the immobilized enzyme at various incubation periods (4, 8 and 12 h) in which lane A, B are the standards and lane C is the acid hydrolysis product.

been shifted to 224 °C after amination step. This improvement could be attributed to a complex network formed between gel beads and PEI. After activation with GA; the temperature increased gradually to three peaks at 255, 320 and 436 °C. This may be attributed to further cross-linking between GA and aminated gel beads. Finally, after immobilization of enzyme, three bands appeared at higher temperature 246, 309 and 895 °C. These finding is matching with the previously published data (Elnashar and Hassan, 2014).

3.5. Scanning electron microscope

The examination of the surface of different beads formulations was performed under scanning electron microscope that showed a characteristic difference in the surface after each step (Fig. 4). The surface of the beads (A) was highly irregular, the addition of PEI (B) cover all the surface but the surface appearance still coarse, in (C) the GA particles were noticed and finally the immobilized enzyme cover all the surface as seen well in (D). Similar result was achieved by Hashem et al. (2016).

3.6. Temperature and pH

Measurements of the immobilized chitinolytic activity as a function of the temperature of the reaction indicated that the activity of the enzyme increased up to 60 °C (Fig. 5A). After this point, the enzyme activity decreased that may be attributed to the thermal denaturation of the enzyme. An important finding was the better performance of the immobilized enzyme at temperatures up to 60 °C in compare to 55°C reported in a previous study for the free enzyme (Ismail et al., 2019). This finding is similar to that reported by El-Shora et al. (2018) and Preety and Hooda (2018) but higher than the optimum temperature (50 °C) observed by the immobilization of chitinases reported by Prasad and Palanivelu (2014) and 45 °C reported by Esawy et al. (2016). The shift in the enzyme optimum temperature that was observed after immobilization, may be attributed to the formation of a molecular cage around the enzyme, protecting it from the mild denaturation temperature (Esawy et al., 2016 and Preety and Hooda, 2018).The optimum pH of the immobilized enzyme using 0.05 M acetate buffer was pH 5 (Fig. 5B) at which the enzyme retained 100% of its activity up to 2 h (Fig. 5C).The optimum activity of the immobilized chitinase in acidic conditions was reported by other researchers (Esawy et al., 2016; Prasad and Palanivelu, 2014; Preety and Hooda, 2018) while El-Shora et al. (2018) reported that the optimum pH of the immobilized chitinase was 8–9.

3.7. Arrhenius plot of the immobilized enzyme

Arrhenius plots are always used to study the effect of temperature on the rates of the reactions. So by plotting of ln k (ln relative activity %) versus the reciprocal of the temperature in Kelvin, a straight line was observed in which the slope represented the value of $-E_a/R$. E_a is the activation energy and R is the gas constant. The E_a of the immobilized enzyme was calculated to be $20.287 \pm 0.159 kJmol^{-1}$. El-Shora et al. (2018) reported activation energy ranging from 11.6 to 30.8 kJ mol^{-1} for chitinase immobilized on various carriers while Preety and Hooda (2018) reported an activation energy of $3.32 kJmol^{-1}$ for the immobilized chitinase. The lower the *Ea* value indicated the lower energy required for the conformation of the active site for the enzyme substrate complex that reflected to a decrease in the total cost of the enzyme.

3.8. Kinetic constants for the immobilized enzyme

The activity of the enzyme was determined at different substrate concentrations. The maximum activity of the immobilized enzyme was observed by using substrate concentration of 2 mg/mL (Fig. 6A) in compare to 1 mg/mL for the free enzyme reported previously by Ismail et al. (2019). The kinetic constants for the immobilized enzyme were calculated on the base of Lineweaver-Burk plot (Fig. 6B). K_m and V_{max} were calculated to be 2 mg/mL and 5.9U/mg protein/min respectively. Esawy et al. (2016) reported 2.0 mg/mL and 40.0U/mg/min as the K_m and V_{max} values for the immobilized *Aspergillus awamori* EM66 chitinase. Preety and Hooda., (2018) reported K_m and V_{max} values of 1.63 mg/mL and 980.39 µmol (min mg protein)$^{-1}$ for immobilized chitinase. The K_m and V_{max} values depend mainly on the enzyme source reflecting the sensitivity of the enzyme toward the substrate i.e. the

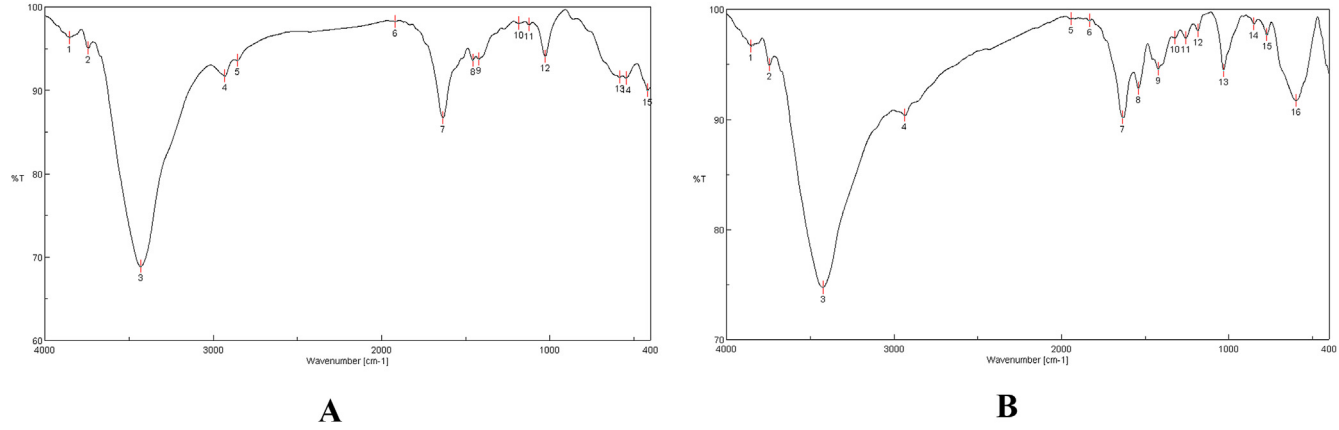

Fig. 10. FTIR analysis of (A) the final hydrolysis product of chitin (after 12 h hydrolysis period) using the immobilized enzyme (B) standard glucosamine hydrochloride.

decrease in the K_m and the increase in V_{max} indicate the increase in the enzyme sensitivity toward the substrate (Horn et al., 2006).

3.9. Thermal stability

Incubation of the immobilized enzyme at temperature range from 45 to 65 °C for time interval from 15min up to 2 h indicated that the immobilized enzyme presented thermal stability at temperatures up to its optimal temperature activity (60 °C) (Fig. 7). This thermal stability may be due to the restricted conformational changes of the enzyme attributed by the immobilization process. Similar results were observed by Esawy et al. (2016), Fernandes et al. (2013) and Preety and Hooda (2018). On the other hand, the chitinase produced by *Alternaria infectoria* retained about 40% of its activity after 2 h at 60 °C (El-Shora et al., 2018). This is a very important finding, since an important factor to be considered in the development of large-scale industrial method for polysaccharide hydrolysis is the temperature of the reaction. High temperatures are preferred in order to control microbial contamination.

3.10. Enzyme reusability

The enzyme reusability depends on two main parameters: storage stability and repeated use, which are commonly named operational stability. The results of the repeated use of the immobilized enzyme indicated that it kept its complete activity for more than 15 cycles. From the economic point of view, it is a very important finding as it will reduce the total cost of the enzyme. Moreover, the immobilized enzyme kept its complete activity at 4 °C for more than one month otherwise it dried. The high operational stability of the immobilized enzyme makes it suitable for the large-scale industrial use. Fernandes et al. (2013) reported that the totally cinnamoylated derivative of D-sorbitol-chitinase beads retained 83% of its initial activity at the 14th cycle. Preety and Hooda (2018) reported that polyurethane/nano ZnO bound enzyme could be reused 10 times without any appreciable loss of activity while Seo et al. (2012) reported that the chitosan-chitinase beads retained 55% of its initial activity after 2 cycles.

3.11. Hydrolysis of chitin

Initially, the chitin hydrolysis activity of the immobilized enzyme was evaluated by determining the amount of the reducing sugars released. The results shown in figure (8) indicated that the amount of the released reducing sugar was 58.86 μg/mg chitin after 1 h incubation period and increased to reach 193.8 μg/mg chitin after 12 h. After that, no increase in the amount of the released reducing sugar was observed.

The hydrolytic pattern was observed by TLC analysis (Fig. 9). The result indicated that the hydrolytic product of crab chitin by using the immobilized enzyme different from that obtained by acid hydrolysis. Moreover, the TLC analysis indicated that the degree of polymerization of the resulted hydolyzate decreased with increasing incubation period up to 12 h but not reached the constituent monosugar of chitin (N-acetyl glucosamine) or its deacetylated derivative (glucosamine). The final degradation product was analyzed by FTIR (Fig. 10). The spectrum shows similarities with that of a standard glucosamine hydrochloride that shows characteristic absorption bands at wave length 3423.99 cm^{-1} derived from O–H stretching vibration merged with that of N–H stretching band and at 1634.38 cm^{-1} of the amide bond, indicating the de-acetylating activity of the immobilized enzyme. The enzymatic hydrolysis of chitin provides an eco-friendly approach that attracts the research focus. Several reports reported the use of free chitinases for the hydrolysis of chitin and production of N-acetyl glucosamine (Cardozo et al., 2017; Das et al., 2019; Zhang et al., 2018) while Kidibule et al. (2018) reported COS production of 150 μg/mg colloidal chitin using free chitinase. The use of immobilized enzymes for the hydrolysis of chitin and production of COS was at most negligible.

4. Conclusion

The use of carrageenan in addition to guar gum in the ratio 2:1 forms an efficient carrier for the immobilization of *Alternaria* sp. strain Sha exochitinase that possesses thermo activity and thermo stability. The immobilized enzyme can be used for more than 15 cycles that will increase its economic viability. In the current study the high optimal activity in addition to the high thermal and operational stabilities of the immobilized enzyme collectively makes it a suitable candidate for large-scale industrial applications. The immobilized enzyme can be used directly for the hydrolysis of chitin and production of COS.

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