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Kinetic, Thermodynamic and Bio-applicable Studies on *Aspergillus niger* Mk981235 Chitinase

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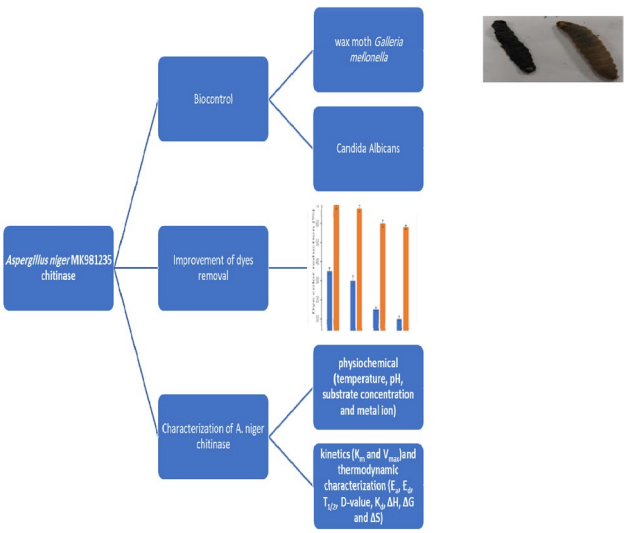
Abstract

Chitinases have many applications in food, agricultural, medical, and pharmaceutical fields. This study succeeded in investigating *Aspergillus niger* MK981235 chitinase in the spot of its physiochemical, kinetic, thermodynamic, and application. The optimum temperature, pH and p-nitrophenyl-β-D-N-acetyl glucosaminide (PNP-β-GlcNAc) concentration to obtain the highest chitinase activity of 2334.79 U ml^{-1} were at 60 °C, 5 and 0.25%, respectively. The kinetic parameters, including K_m and V_{max} were determined to be 0.78 mg ml^{-1} and 2222.22 µmol ml^{-1} min^{-1}, respectively. Furthermore, the thermodynamic parameters $T_{1/2}$, D-values, ΔH, ΔG and ΔS at 40, 50 and 60 °C were determined to be (864.10, 349.45, 222.34 min), (2870.99, 1161.07, 738.74 min), (126.40, 126.36, 126.32 kJ mol^{-1}), (101.59, 100.62, 100.86 kJ mol^{-1}), (74.50, 76.17, 47.24 J mol^{-1} K^{-1}), respectively. *A. niger* chitinase showed, insecticidal activity on *Galleria mellonella* by feeding and spraying treatments (72 and 52%, respectively), anti-lytic activity against *Candida albicans*, and effectiveness in improving the dye removal in the presence of crab shell powder as bio-absorbant. *A. niger* chitinase can be used in the pharmaceutical field for the bio-control of diseases caused by *C. albicans* and for the pretreatment of wastewater from the textile industry.

Graphical Abstract



Keywords Chitinase · Thermodynamics · Bioapplicability

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1 Introduction

Rapid progress in the industrial and agricultural fields has led to an increased demand for applicable enzymes. Chitinase is one of those enzymes that has numerous applications. Chitinases (EC 3.2.1.14), which hydrolyze chitin to release GlcNAc and N-acetyl chito-oligosaccharides (COSs), are produced by a wide range of organisms, including viruses, bacteria, fungi, insects, higher plants, and animals [1]. Chitin is a carbohydrate polymer composed of N-acetyl D-glucosamines connected by β-1,4-glycosidic linkages [2]. Chitin is present in nature in the structure of the exoskeleton and the shell of crustaceans as well as in the cell walls of fungi.

Chitinases are required in the agricultural field, for biological control of fungal pathogens and as pesticides against insects [3–5]. Biological control is more favorable than chemical one since the long-term use of the latter one has harmful effects on the environment and human health. In the energy industry for bioethanol production [6], In the medical and pharmaceutical fields as an antitumor agent, and in the preparation of ophthalmic solutions [7]. It is also demanded in the food industry to increase tannase release from the cell wall of fungi [8]**.** The products of chitinase activity, including N-acetyl-D-glucosamine [9] and N-acetyl chito-oligosaccharides, are also required in the food, medicinal, and biotechnology sectors for having activity as prebiotics [10], anti-oxidants, and anti-inflammatory mediators [11]**.**

In recent years, the accumulation of large quantities of shellfish waste from shrimp, crabs, and krill has been paid attention as a source of 20–30% chitin [7]. The conversion of these chitinous wastes into applicable products can be achieved chemically or biologically, but the former usually leads to low production efficiency and environmental pollution [12].

For dye removal, activated carbon is the most commonly used sorbent, but due to the high cost and difficulty of regeneration [13], there was a necessity for searching for alternatives. At this point, it was found that fishery wastes that contain chitin or chitosan can perform this mission successfully as a cheap and effective new choice after biological treatment [14].

Therefore, in this study, we investigated the physiochemical, kinetic, and thermodynamics of *Aspergillus niger* MK981235 chitinase produced utilizing molokhia stems as nutritional substrate. The anti-yeast activity of *Aspergillus niger* MK981235 chitinase against *Candida albicans* was also investigated. Also, the effectiveness of *A. niger* chitinase on dyes removal improvement was evaluated.

2 Expermintal

2.1 Chitinase Production

Aspergillus niger MK981235 chitinase was prepared by cultivating *A. niger* by the solid state fermentation (SSF) technique on molokhia stems (MS) as described previously [15].

2.2 Chitinase Assay

It was detected utilizing p-nitrophenyl-β-D-N-acetyl glucosaminide (PNP-β-GlcNAc) as a substrate, and measuring N-acetyl glucosamine produced as a product with dinitrosalicylic acid (DNSA) method as described by Matsumoto et al. [16].

2.3 Characterization of *Aspergillus niger* MK981235 Chitinase

The conditions for maximum chitinase activity were investigated by performing the reaction at different temperatures (30, 40, 50, 60, and 70 °C), pH (4, 5, 6, and 7) and PNP-β-GlcNAc concentrations (0.0125, 0.025, 0.05, 0.1, 0.15, 0.2, 0.25, and 0.3%). Also, the effect of different metal ions (Na^+, K^+, Cu^{2+}, Zn^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+}, Ni^{2+}, Co^{2+}, and Ba^{2+}) with a final concentration of 5 mM was investigated by pre-incubating them with the enzyme at 30 °C for 30 min, followed by performing the reaction at optimum conditions and considering the chitinase activity in the absence of metal ions as 100%. The thermal stability of *A. niger* chitinase was investigated by pretreatment at different temperatures (60, 65, and 70 °C) for various periods (15, 30, 45, and 60 min).

2.4 Kinetic and Thermodynamic Characterization

The kinetics, including K_m (Michael's constant) and V_{max} (maximum velocity), were determined from the Lineweaver–Burk plot. The E_a (activation energy) and E_d (energy of denaturation) were calculated from the Arrhenius plot.

$$\text{Slope} = -E_d/R.$$

The thermodynamics $T_{1/2}$ (half-life), D-values (decimal reduction time), ΔH_d (change in enthalpy), ΔG_d (free energy), ΔS_d (entropy) were determined from the following equations as described by Abdel Wahab et al. [17]:

$$T_{1/2} = \ln 2/K_d.$$

$$\text{D-value} = \ln 10/K_d.$$

$$\Delta H = E_d - RT.$$

$$\Delta G = -RT \cdot \ln\left(K_d \cdot h/K_b \cdot T\right)$$

$$\Delta S = \left(\Delta H_d - \Delta G_d\right)/T.$$

where T is the corresponding absolute temperature (K), R is the gas constant (8.314 J mol^{-1} K^{-1}), h is the Planck constant (6.626×10^{-34} J min), K_b is the Boltzman constant (1.38×10^{-23} J K^{-1}) and K_d is the deactivation rate constant (min^{-1}).

2.5 Anti-yeast Activity

Candida albicans was used to investigate the yeast lytic activity of *A. niger* chitinase as described by Karthik et al. [18]. This was done by incubating potato dextrose agar (PDA) plates inoculated with 0.1 ml yeast cell suspension (10^7 spores ml^{-1}) and pored with wells containing 200 µl chitinase (191 U) at 30 °C.

2.6 Dye Removal Enhancement by *A. niger* Chitinase

In this experiment, *A. niger* chitinase (382.86 U) in the presence of crab shell powder (1 g, untreated) were mixed with 0.1% dye (crystal violet, brilliant blue, brilliant green, methylene blue) and the reduction in color intensity was measured spectrophotometrically at 420 nm.

2.7 Insecticidal Activity

The insecticidal activity was tested on larvae of the larger wax moth, Galleria mellonella, in their sixth instar (Lepidoptera: Galleridae). Galleria stock cultures were collected from infested hives and raised in jars (2 kg capacity) containing a specific medium made up of wheat (130 g), wheat bran (130 g), milk powder (130 g), maize flour (97.5 g), yeast powder (97.5 g), wax (26 g), honey (195 ml), and glycerol (195 ml) until moths appeared.

3 Results and Discussion

3.1 Characterization of *A. niger* Chitinase

As shown in Fig. 1a *A. niger chitinase was active over a wide temperature range of* 30–70 °C, emphasizing its usefulness in a variety of industrial fields. Its maximal activity was recorded at 60 °C (956.70 U ml^{-1}), which was similar to *Cohnella sp*. A01 chitinase [19]. According to Vincy et al. [20] for *Vibrio alginolyticus* at 45 °C, Abdel Wahab et al. [17] for *Trichoderma longibrachiatum* KT693225 at 40 °C, and Subramanian et al. [21] for *Achromobacter xylosoxidans* at 45 °C, most chitinases have their maximal activity around 40 °C.

A. niger chitinase was almost unaffected by heat pretreatment at 60 °C for 60 min, retaining 95.41% of its initial activity, as shown in Fig. 1b demonstrating its high thermal stability. Thermal pretreatment of *A. niger* chitinase at higher temperatures (65, and 70 °C) for various periods of time caused gradual decrease in enzyme activity due to protein denaturation. The activity of *A. niger* chitinase peaked at pH 5 (956.70 U ml^{-1}) and then declined drastically below and above, as reported by Aliabadi et al. [19] and Dai et al. [22]. Vincy et al. [20] and Subramanian et al. [21] found that pH 9 and 8 were the best for chitinase from *Vibrio alginolyticus* and *Achromobacter xylosoxidans*, respectively. With 0.25% PNP—GlcNAc, the maximal *A. niger* chitinase activity of 2334.79 U ml^{-1} was obtained, after which any substrate increase had no effect on enzyme activity (data not shown) and this may be due to the full saturation of enzyme active sites with the substrate. The activity of *A. niger* chitinase, as shown in Fig. 1c, was unaffected by any of the metal ions tested. In contrast, they had a variable inhibitory effect on chitinase activity, with Hg^{2+} causing a 72% drop in activity. In addition, Cu^{2+} and Co^{2+} reduced activity by 52.6 and 35.6 per%, respectively, as reported by Dai et al. [22]. Aliabadi et al. [19] found that Cu^{2+} had a favorable effect on *Cohnella* sp. A01 chitinase.

3.2 Kinetics and Thermodynamics Characterization of *A. niger* Chitinase

It's crucial to understand the kinetics and thermodynamics of every enzyme before deciding whether it's suitable for industrial use. The K_m and V_{max} values are important factors that determine the enzyme's sensitivity to the substrate. K_m and V_{max} were found from the Lineweaver Burk plot (Fig. 2a) to be 0.78 mg ml^{-1} and 2222.22 mol ml^{-1} min^{-1}, respectively. K_m for chitinases from *Cohnella* sp. A01 and *T. longibrachiatum* were determined to be 5.6 mg ml^{-1} [19] and 8 mg ml^{-1} [17], respectively, due to the strong affinity of chitinase for the PNP—GlcNAc.

E_a, E_d (Fig. 2b, c), $T_{1/2}$, K_d, D-value, ΔH, ΔG, and ΔS are some thermodynamic characteristics that characterize the stability of the enzyme (Table 1). At 60 °C, the half-lives of *A. griseoaurantiacus* KX010988 [23] and *T. longibrachiatum* KT693225 [17] were 205.63 and 220.64 min, respectively, compared to 864.10 min for *A. niger* chitinase. The *A. niger* chitinase stability is highlighted by the low E_a (3.87 kJ) and high E_d (129.11 kJ mol^{-1}) values (Fig. 2b, c). The lower the E_a value, the less energy is required to produce the active complex (enzyme–substrate), and the higher the E_d value, the more energy is required to denaturate the enzyme [17]. E_d value for *A. niger* chitinase (129.11 kJ mol^{-1}) reflected

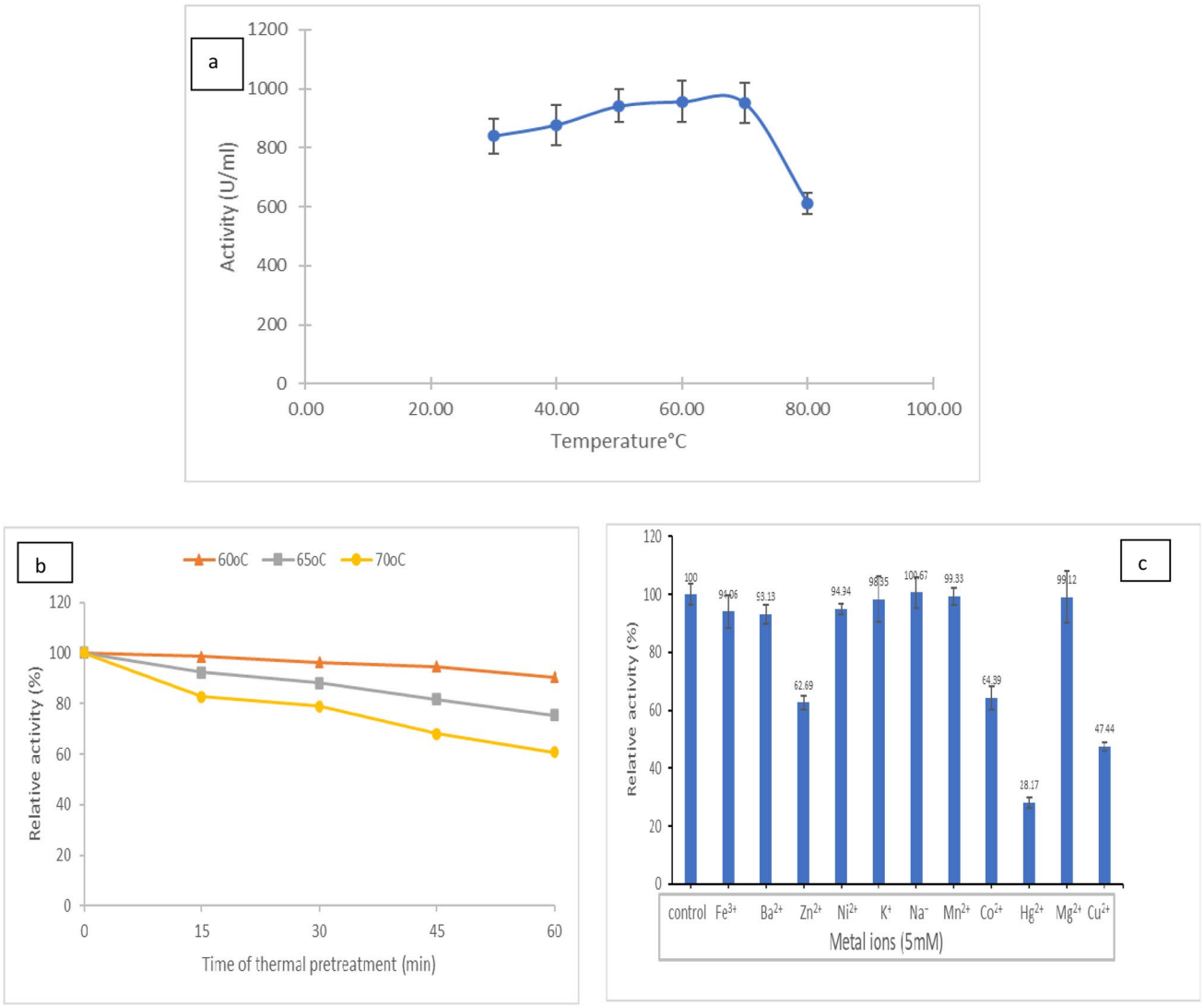


Fig. 1 Physiochemical characterization of *A. niger* chitinase, effect of, **a** reaction temperature; **b** thermal pretreatment; **c** metal ions on chitinase activity

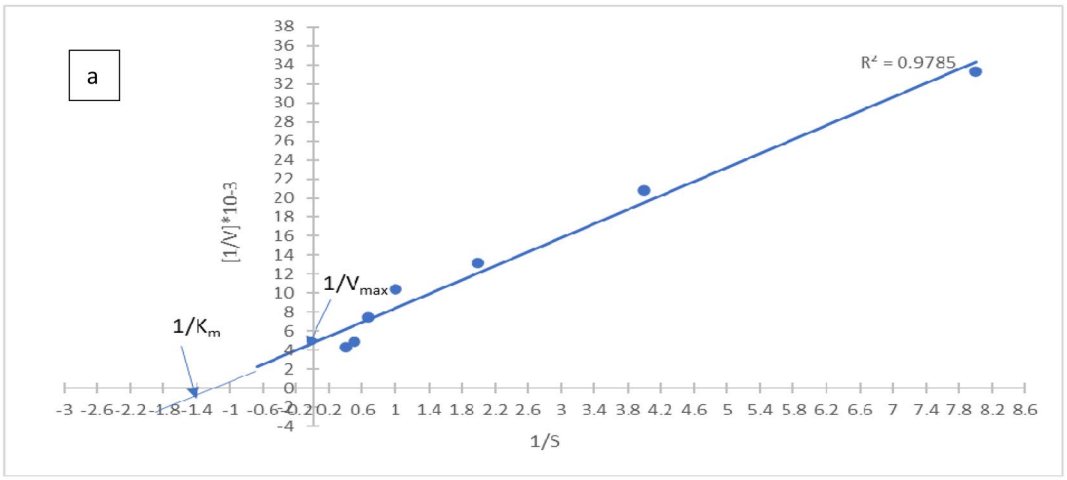
the higher thermostability than those for *A. griseoaurantiacus* KX010988 (50.72 kJ mol^{-1}) [23] and *T. longibrachiatum* KT693225 (28.87 kJ mol^{-1}) [17] meaning that *A. niger* chitinase required more energy for denaturation.

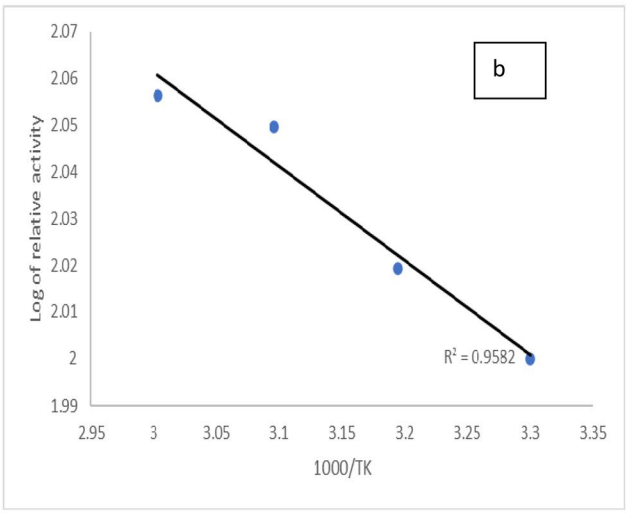
3.3 *Aspergillus niger* Chitinase Anti-yeast Activity

The majority of the identified chitinases have antifungal activity against *Fusarium oxysporum*, *Trichoderma viride*, *Aspergillus oryzae*, *Penicillium oxysporium*, *Rhizocotonia solani*, *Fusarium solani,* and *Colletotrichum* sp. [4, 18, 24–27]. *Candida albicans* causes superficial mucosal candidosis and a variety of severe infections [28], and its hyphal development is critical for virulence [29, 30]. As a result, the most effective treatment should target hyphal morphogenesis rather than pathogen survival. *A. niger* chitinase showed antimicrobial activity against *Candida albicans* (3 cm). Due to the presence of chitinase activity, Farag et al. [26] and Allonsius et al. [31] found antimicrobial action for *A. terreus* and *Lactobacillus rhamnosus* GG, respectively, against *C. albicans*. Streptomyces sp. chitinase, on the other hand, had no effect on *C. albicans* growth [18].

3.4 Dyes Removal

Figure 3 revealed some observations, First, there was a variance in dye color reduction (10–70%) depending on the dye, and second, the presence of chitinase improved dye reduction. Brilliant blue had the biggest drop in dye intensity (70%) in the presence of chitinase and crab shell powder, compared to just 35% in the presence of crab shell powder. For dye removal, Liang et al. [14] used squid pen





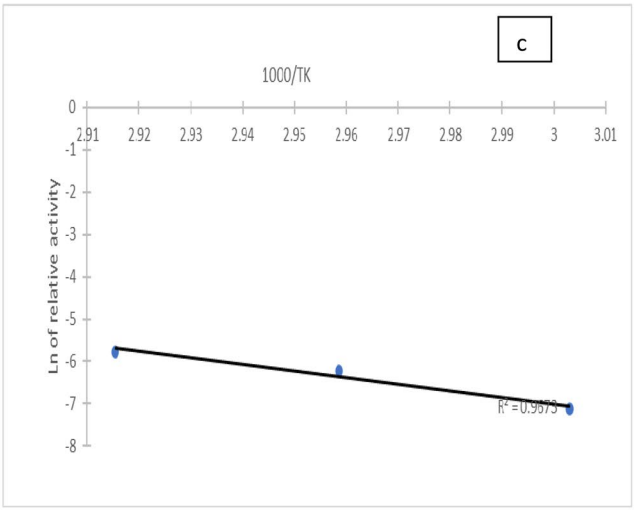


Fig. 2 Kinetic and thermodynamic characterization of *A. niger* chitinase, **a** Detremination K_m and V_{max} from Lineweaver Burk-Plot; Arrhenius plot for determining, **b** activation energy E_a and **c** activation energy of denaturation E_d for *A. niger* chitinase

Table 1 Thermodynamic of denaturation of *A. niger* chitinase

Temperature (°C)	K_d (min)	$T_{1/2}$ (min)	D-value (min)	ΔH (KJ mol^{-1})	ΔG (KJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
60	8.02×10^{-4}	864.10	2870.99	126.40	101.59	74.50
65	19.84×10^{-4}	349.45	1161.07	126.36	100.62	76.17
70	31.17×10^{-4}	222.34	738.74	126.32	100.86	74.24

powder fermented with chitinolytic bacteria and found that color reduction was more noticeable in the presence of fermented squid pen than in the presence of unfermented squid pen. According to Laing et al [14], color adsorption into chitinous waste occurs by physical adsorption in fermented waste and chemical adsorption in unfermented waste.

The presence of functional groups such as amino and hydroxyl groups serving as dye-binding material could explain the dye removal action.

3.5 Insecticidal Activity

Insect control can be achieved by changing their peritrophic membrane, which protects the midgut epithelium

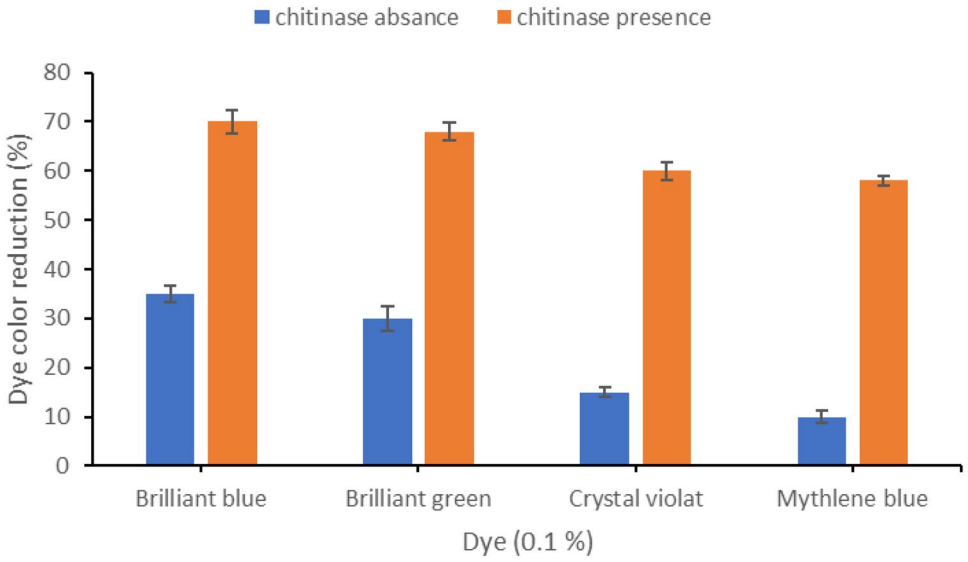


Fig. 3 Improvement effect of *A. niger* chitinase on dyes removal in the presence of crab shells powder as bioabsorbant

and hence reduces their feeding [32]. Table 2 shows that mixing Galleria's food with *A. niger* chitinase or spraying it with *A. niger* chitinase resulted in mortality rates of 72 and 52%, respectively. Galleria's chitin polymer was rapidly depolymerized by the chitinase enzymes, resulting in chitin breakdown and the pest's death. Bahar et al. [32] discovered a substantial link between bacterial isolates' insecticidal and chitinase activity. Insecticidal actions of chitinases on Galleria mellonella were reported by Awad et al. [33] and Abulikemu et al. [34].

4 Conclusion

The physiochemical, kinetics, and thermodynamics of *A. niger* MK981235 chitinase highlighted its thermostability and the prospect of its use in industrial applications. The activity of *A. niger* against *C. albicans* allows it to be utilized in the biocontrol of *C. albicans*-related disorders, which is more effective than chemical treatment. The improved dye removal in the presence of *A. niger* chitinase with chitinous waste further suggests that it could be used in the biotreatment of textile industry wastewater.

Table 2 Insecticidal effect of *A. niger* MK981235 chitinase showing mortality percentages of the greater wax moth larvae *Galleria mellonella* after feeding or spraying treatment

Replicates	Feeding					Contact				
	Larvae		Pupae		Adults	Larvae		Pupae		Adults
	Dead	Live	Dead	Live		Dead	Live	Dead	Live	
R1	3	1	1	0	0	3	1	0	1	0
R2	2	0	2	0	0	1	2	1	1	0
R3	3	0	1	1	0	3	1	0	1	0
R4	4	0	0	1	0	2	3	0	0	0
R5	2	1	0	1	0	2	0	0	1	0
R6	3	0	0	1	0	2	0	1	0	0
R7	2	1	1	0	0	3	1	0	1	0
R8	3	0	1	1	0	2	2	0	1	0
R9	3	0	0	1	0	3	0	0	0	0
R10	3	0	2	0	0	2	2	1	0	0
Total	28	3	8	6	0	23	12	3	6	0
% Mortality	56		16			46		6		
Total % Mortality	72					52				

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Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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