https://doi.org/10.22319/rmcp.v16s4.6692

Article

Assessment of the coleoptericidal potential of crude extracts of ChiBLUV 02 recombinant chitinase of *Bacillus licheniformis* on *Aethina tumida*

Deny de Jesús Velasco-Vique [a]

Argel Flores-Primo [a]*

Rodolfo Quintana-Castro [b]

Violeta Trinidad Pardío Sedas [a]

María Guadalupe Sánchez-Otero [b]

Anabel Cruz Romero [a]

[a] Universidad Veracruzana. Facultad de Medicina Veterinaria y Zootecnia. Miguel Ángel de Quevedo S/N, 91710, Veracruz, Ver, México.

[b] Universidad Veracruzana. Facultad de Bioanálisis, Veracruz, Ver, México.

*Corresponding author: argflores@uv.mx

Abstract:

Aethina tumida is a pest that causes structural and production damage in the beekeeping industry. The control method includes using synthetic pesticides that can affect bees, contaminate honey and other hive products, and generate resistance to pests. The present study assessed the coleoptericidal effect of a crude extract of ChiBLUV 02 recombinant chitinase of *Bacillus licheniformis* UV01 expressed in *Escherichia coli* BL21 (De3) strains on the species *Aethina tumida*. Different units of enzymatic activity (0.42, 1.26, 2.10. 4.20, 8.40, 12.60, 16.80, and 21.00 U/ml) mixed with the maintenance food for larvae and beetles were evaluated. The food was provided in portions of 1 g/d for 3 days, and the coleoptericidal effect was evaluated at 24, 48, and 72 h. The LC_{50} and LC_{90} were calculated using a Probit analysis. Applying 21.00 U/ml of recombinant chitinase promoted mortality of 45 % of larvae after 72 h of administration; nevertheless, none of the concentrations evaluated affected adult

beetles. The Probit analysis indicated that 27.03 and 168.92 U/mL are needed to promote mortality of 50 % (LC_{50}) and 90 % (LC_{90}) of *Aethina tumida*. The enzymatic activity of the crude extracts was low to achieve higher mortality of the larvae and adults of *Aethina tumida*; therefore, the chitinolytic activity in the ChiBLUV 02 strains should be improved to increase their coleoptericidal effect.

Keywords: Beetle, Larvae, Biopesticide, Enzyme, Probit.

Received: 22/05/2024

Accepted: 12/01/2025

Introduction

Beekeeping is an underlying activity of the livestock sector that provides raw materials for the national and international food, pharmaceutical, and cosmetic industries. In addition, the beekeeping sector provides economic income for many families[1]. Nonetheless, one of the big problems of beekeeping is the poor practices of management and storage of products derived from the hive by beekeepers, which favors the presence of pests and causes economic losses. One of the main pests affecting the beekeeping sector is *Aethina tumida*, a species known as the "small hive beetle" that could be classified as a bee parasite due to how it develops at the expense of bees inside the hive. This species is native to the tropical and subtropical regions of sub-Saharan Africa[2], but in the last 20 yr, it has gained international importance as it has spread its presence to different parts of the world, causing infestations of bee colonies, mainly *Apis mellifera scutellata* and *Apis mellifera capensis*[3].

The main impacts of *A. tumida* on beekeeping include structural damage to the hive and the fermentation of honey by adult and larval droppings, which directly affects the hive record and the yield and quality of the honey obtained[3,4]. When *A. tumida* infestation in hives is extreme, it can trigger a decrease in the bee population because the adults feed on bee hatchlings, pollen, and honey; these factors, added to the presence of other parasites or diseases, can trigger the collapse of the hive[4]. This species is controlled by several methods, including the strengthening of bee colonies and good beekeeping management practices, the use of traps, as well as the use of pesticides, such as coumaphos (CheckMite+™) and fluvalinate, which are used inside the hive, or permethrin, which is applied to the soils around the hives. Synthetic pesticides are effective in controlling this species; however, their use can have serious consequences that lead to the contamination of honey and other products of the

hive, damage to the environment, alterations in the bee population, damage to the health of beekeepers, as well as the generation of resistant pests[5].

The current trend has focused on the development of biopesticides from natural sources, the application of enzymatic biotechnology, or the use of natural predators or parasitoids of the species. One of these alternatives is the use of chitinases, highly specific enzymes that hydrolyze chitin, a carbohydrate that is present in 75 % of the exoskeleton of insects, which gives them strength and rigidity[6], which could be used in combination with traps inside hives that allow pests to enter and avoid contact with bees. In addition, some authors have mentioned that chitinases promote an antifeedant effect in insect larvae because they affect the structure of the peritrophic membrane of their intestines, promoting a decrease in pupation success and increasing mortality in the larval and pupal stages[7,8]. Bacteria of the genus *Bacillus* are one of the largest sources of biologically active natural compounds[9], which produce numerous secondary metabolites and a large number of enzymes; likewise, this genus contains some species called harmless and favorable for crops and the environment, such as *B. subtilis*, *B. licheniformis*, and *B. pumilus*; nevertheless, before their use in the field, they must be extensively evaluated in terms of biosafety[10]. Several *Bacillus* species show chitinolytic activity, such as *Bacillus pumilus*[11], *B. licheniformis* strain LHH100[12], and *B. licheniformis*[13,14]. Because chitinases hydrolyze chitin, a polysaccharide found in high proportions in insects, fungi, yeasts, and internal structures of some vertebrates, several studies have focused on the use of these enzymes for the biological control of pathogens that affect plants and insect pests that affect the agricultural sector[14,15,16].

At the moment, there are no studies that have proven the effectiveness of chitinases on *A. tumida*, but due to the high percentage of chitin that its exoskeleton contains[6] and the food effect that has been reported in other studies, it is considered that the application of chitinases[7] could be a viable alternative for its control. Nonetheless, bees also have a high percentage of chitin in the composition of their exoskeleton and could be indirectly affected during its application, so if the treatment is effective, in the following studies, it would be proposed to evaluate different methods of application which could combine the use of chemical attractants (pheromones) and traps that allow the entry of larvae or adults of *A. tumida* but not bees to avoid affecting the hive. Therefore, the present work is the first of a series of studies that aims to generate the bases for the development of an effective and natural treatment for the control of *A. tumida* by determining the coleoptericidal effect of a chitinase obtained from *B. licheniformis* UV01 and expressed in *Escherichia coli* BL21 (DE3) under laboratory conditions.

Material and methods

The strains of *Escherichia coli* BL21 (DE3) that produce the recombinant chitinase were obtained from the strain collection of the chemistry and genomics laboratory of the Faculty of Bioanalysis, Veracruz campus. To obtain the crude extract of recombinant chitinase, the *Escherichia coli* BL21 (DE3) strains were pre-inoculated on Luria Bertani (LB) agar added with Kanamycin (10 mg/ml) to obtain an exponential growth of cell culture. A culture of 50 mL of LB medium was performed by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG) to induce the expression of the protein of interest (chitinase), leaving incubation for 8 h at 37 °C and stirring at 220 rpm. To obtain the enzymatic crude extract, the culture was sonicated for 20 cycles of 15 sec with a 30-sec rest and subsequent centrifugation at 17,200 xg for 15 min and 4 °C[17]. The crude extract obtained was stored at 4 °C in hermetically sealed containers until used in the *A. tumida* trials.

The viability of the recombinant enzyme as a natural alternative for controlling *A. tumida* was determined by evaluating its chitinolytic activity. For this, 500 mL of enzymatic crude extract was added to an Eppendorf® tube and incubated at 32 °C for 5 min. Subsequently, 500 mL of 1 % colloidal chitin was added to 100 mM phosphate buffer with a pH of 6.0 and incubated at 32 °C for 1 h. After the reaction, the concentration of reducing sugars was quantified by the 3,5-dinitrosalicylic acid (DNS) method[18] as an indirect measure of the concentration of N-acetyl-D-glucosamine released in the reaction. Enzyme activity was defined as mM of N-acetyl-D-glucosamine released min^{-1} at 32 °C (pH 6). The chitinolytic activity of a crude extract of a non-recombinant strain of *Escherichia coli* BL21 (DE3) was evaluated as a negative control.

Wild *A. tumida* colonies were established with healthy adults (males and females) collected from apiaries in the central area of the state of Veracruz, which were adapted to breeding chambers with a maintenance medium (water and food). The breeding chambers were designed from 60 ×15 mm Científica Senna® Petri dishes, in which a central perforation of 4 cm in diameter was made to promote ventilation. The maintenance medium consisted of administering 1 g of food made with honey, pollen, and beer barley (1:1:1) every third day. Once the beetles oviposited, they were relocated to another Petri dish, and the larvae that hatched were kept with the same food and water conditions for 3 wk and then placed in pupation chambers. Chambers were designed for this stage, which consisted of plastic containers with a capacity of 1 L to which 3/4 parts of sand was added, and a Petri dish with a hole in the base was adapted to the top, which allowed the larvae to descend and start their pupation stage. The new individuals were placed in chambers independent of the first generations and kept with the same water and food conditions. All instars of *A. tumida* development were maintained in a culture oven (Benchtop incubator 12E, Quincy Lab Inc.

Illinois, USA) at 28 ± 2 °C and an average relative humidity of 82 ± 3 % measured with a thermometer-hygrometer (HTC-2 Uplayteck, Mexico). The RH was stabilized by adding a cotton swab with 5 mL of water to the inside of the breeding chambers, which was changed every 48 h. For the trials of coleoptericidal activity, 10-d-old adult beetles with complete metamorphosis (dark in color and completely hardened) were used. For tests in the larval stage, larvae 3 to 4 d after hatching were used[19].

To prepare the different units of activity to be evaluated in the coleoptericidal assay, the activity of the recombinant enzyme was quantified, and from this, 8 concentrations were evaluated (0.42, 1.26, 2.10. 4.20, 8.40, 12.60, 16.80, and 21.00 U/ml). The recombinant chitinase crude extract was mixed with the food and administered to groups of 10 beetles or larvae for each of the concentrations evaluated, and the acute effect was determined at 24, 48, and 72 h. For the control group, maintenance food without enzyme was administered for both instars. Any specimen that did not walk or respond to manipulation with the Luzeren® dissection forceps was considered dead. In all trials, treated beetles and larvae were kept at 28 ± 2 °C with relative darkness. The response variable was the percentage of mortality of beetles and larvae (24, 48, and 72 h after the start of the trial). All trials were conducted in duplicate.

Mortality data were statistically analyzed by an analysis of variance (ANOVA) at a significance level of 0.05 and comparison of means through Tukey's test using the SAS statistical package, version 9.3. Probit analysis was used to calculate the LC_{50} and LC_{90}[20].

Results and discussion

The recombinant chitinase crude extract presented 42 units of enzymatic activity per milliliter (U/ml), which were taken as a basis for calculation to adjust the activity units evaluated on *A. tumida*. The crude extract of the non-recombinant enzyme did not show chitinolytic activity, proving the viability of the recombinant enzyme. Once the food with recombinant enzyme was administered to *A. tumida* larvae and adults, mortality was assessed every 24 h to 72 h. The maximum mortality of *A. tumida* larvae was observed at 48 h of food administration; after that time, there was no larval mortality. Because of this, the calculation of the LC_{50} and LC_{90} was made using the observations obtained during this time.

Coleoptericidal activity of recombinant chitinase crude extract

The results of the coleoptericidal tests on *A. tumida* larvae showed statistically significant differences in the percentage of mortality when different units of enzymatic activity were applied ($P \leq 0.05$). The percentage of mortality increased as enzymatic activity increased, with the best result obtained with 21 U/ml of recombinant chitinase, which promoted 45 % mortality of *A. tumida* larvae (Table 1). The minimum time required to observe the acute effect of the enzyme extracts was 48 h. On the other hand, the coleoptericidal effect could be seen from the addition of 4.2 U/ml of recombinant chitinase, which resulted in 10 % mortality; however, the enzymatic activity in the extract was insufficient to obtain mortalities higher than 45 %. This shows the need to increase enzymatic activity by changing expression vectors or host cells for its expression that increase protein concentration and enhance its chitinolytic activity.

Table 1: Coleoptericidal activity of recombinant chitinase crude extracts in the larval stage of *A. tumida* (U/ml)

	24 h			**48 h**			**72 h**		
	Live	**Dead**	**Mortality (%)**	**Live**	**Dead**	**Mortality (%)**	**Live**	**Dead**	**Mortality (%)**
*Control	20	0	0	20	0	0	20	0	0
0.42	20	0	0	20	0	0	20	0	0
1.26	20	0	0	20	0	0	20	0	0
2.10	20	0	0	20	0	0	20	0	0
4.20	20	0	0	18	2	10	18	2	10
8.40	20	0	0	16	4	20	16	4	20
12.60	20	0	0	14	6	30	14	6	30
16.80	19	1	5	13	7	35	13	7	35
21.00	18	2	10	11	9	45	11	9	45

*Food without recombinant enzyme.

Another aspect to consider is that it was not possible to use a higher amount of enzyme since, being a liquid crude extract, when mixed with the food, it diluted it and made it difficult to administer. In addition, the enzyme must pass through the digestive tract of the beetle in order to perform its action, which can hinder its action. The recombinant chitinase extract had a coleoptericidal effect on *A. tumida* in its larval stage, promoting up to 45 % mortality when using 21.00 U/ml. The assay was replicated with the beetles in their mature stage, but no effect was observed with the enzymatic activities evaluated. One explanation for this result may be the mechanism of digestion of beetles since it is mentioned that some predatory groups carry out their digestion in the crop by means of enzymes from the midgut, including

proteases, enzymes that could limit the action of chitinases[21]. Other authors have mentioned that the digestive tract of beetles changes during their metamorphosis in the pupation stage to adapt to their adult stage, which could condition the effectiveness of recombinant chitinase when mixed with food[22]. These factors, added to the low enzyme concentration and the impossibility of testing a higher enzyme concentration due to the dilution of the food, could be responsible for the zero mortality observed in the adult stage.

Determination of the LC_{50} and LC_{90}

Larval mortality data were used to perform the Probit analysis and calculate the units of chitinolytic activity required to promote mortality of 50 (LC_{50}) (Figure 1) and 90 % (LC_{90}) (Figure 2) of the population, which were 27.03 and 168.93 U/ml, respectively. The coleoptericidal effect of the crude extract of *B. licheniformis* UV01 chitinase confirms its potential use as an alternative method for controlling *A. tumida*.

Figure 1: LC_{50} of the recombinant chitinase crude extract on *A. tumida* larvae

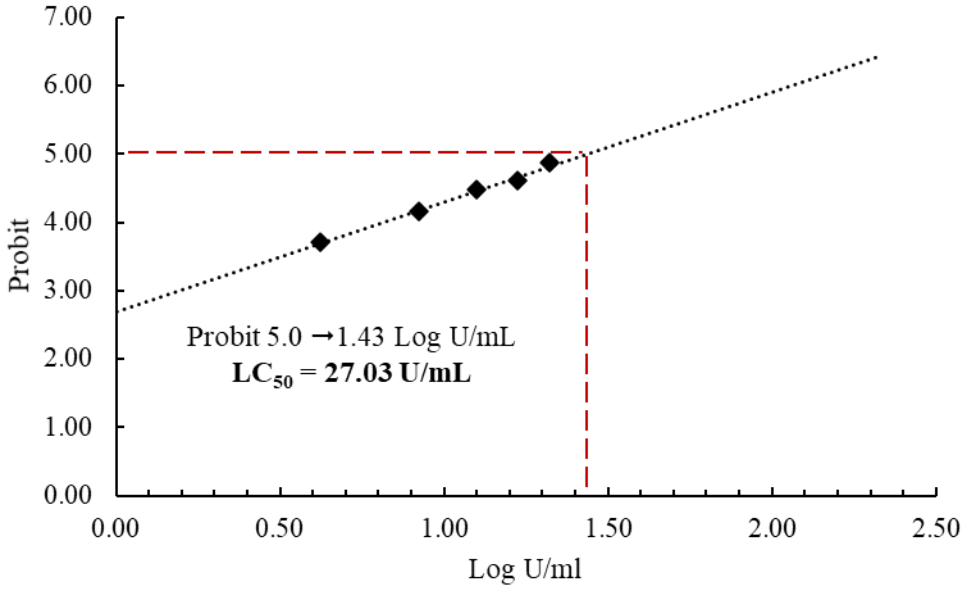
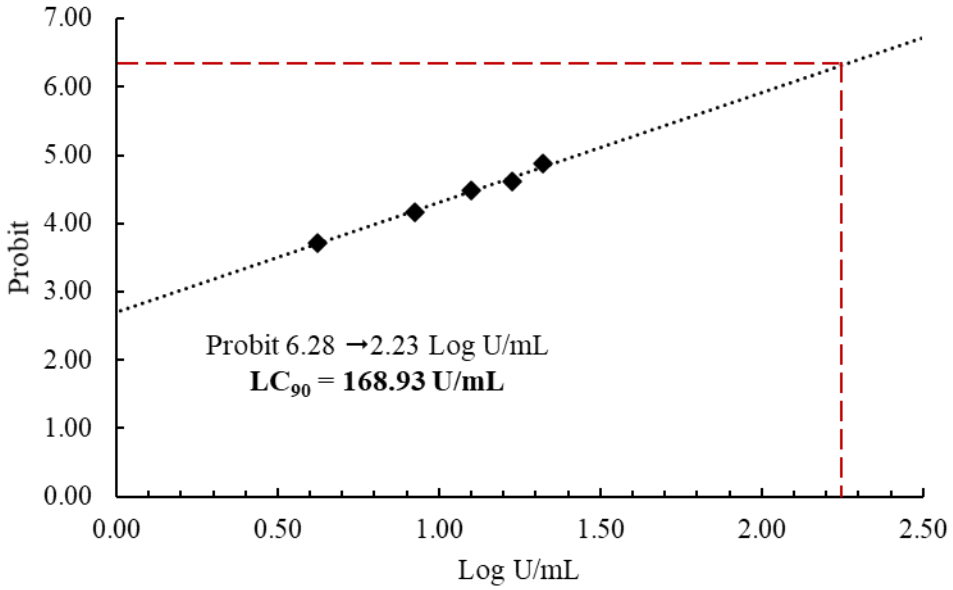


Figure 2: LC_{90} of the recombinant chitinase crude extract on *A. tumida* larvae



These results are comparable with other studies[23,24,25] that have used recombinant chitinases, which have resulted in promising alternatives in pest control.

Discussion

Recombinant chitinases obtained from *B. licheniformis* have been evaluated in several studies with promising results in controlling species that affect the agricultural sector. The crude extract with chitinase of *B. licheniformis* USMW10IK has been assessed in species such as termites *Globitermes sulphureus*, indicating that it has bioinsecticidal properties with a lethality of 23.81 % at 48 h of exposure[25]. Likewise, it has been proposed that the enzymes produced by the genus *Bacillus* are an effective biocontrol alternative against phytopathogens or insect pests through the degradation of the cell wall or cuticles of insects[26]. There are reports of the chitinolytic activity of the genus *Bacillus* against various pests that affect plants and fruits, with an effectiveness of more than 60 %[27,28]. This characteristic suggests that chitinases can be used as a sustainable tool in pest management[28,29].

The present work assessed using a recombinant chitinase of *B. licheniformis* to control *A. tumida*, obtaining outstanding results on the larval stage but without effect on the adult stage. The lack of effectiveness of recombinant chitinase extracts in the adult stage is probably due to the method of application since, when combined with food, the enzyme is exposed to

components that could decrease its activity, added to the feeding mechanism of some predatory beetles that may include proteolytic enzymes, which would affect the activity of chitinase.

Unlike adults, chitin promoted the mortality of up to 45 % of larvae, which may be because, in this stage, *A. tumida* presents notorious differences since it consumes its food in liquid form, secreting enzymes to its food before ingesting it[21], it has a higher feeding rate due to the nutritional and energy requirements it needs to carry out its final metamorphosis, and it has been mentioned that chitinases can promote alterations in the peritrophic membranes of the intestines of larvae that would trigger an antifeedant effect and increased mortality[7]. This was reflected in the results of larval mortality, which increased as the units of activity of the recombinant enzyme increased. This result is remarkable since a crude extract of the enzyme was used, which presented 42 U/ml, which would mean that its chitinolytic activity can be improved by using different expression vectors or host cells that increase the production of the enzyme, as well as by optimizing the fermentation conditions and the components of the culture medium[29].

Conclusions and implications

The potential of crude extracts of recombinant chitinase in controlling pests in the agricultural sector is promising due to their insecticidal effect. In addition, their sustainable use in the control of species such as *A. tumida* would possibly reduce the damage caused by synthetic pesticides, such as pollution of the environment and contamination of bees and products derived from the hive, as well as the damage to the health of beekeepers. However, it is essential to continue with studies to improve their chitinolytic activity, evaluate application methods that promote higher coleoptericidal activity, identify the biosecurity window in which the bee is not affected, and establish strategies for their application in hives through the combined use of traps or devices in which only beetles can enter.

Acknowledgments

To the National Council of Humanities, Science, and Technologies (CONAHCYT, for its acronym in Spanish) for the scholarship awarded to the first author.

Conflicts of interest

None of the authors has a conflict of interest.

Literature cited:

1. Baena-Díaz F, Chévez E, De La Merced FR, Porter-Bolland L. *Apis mellifera* en México: producción de miel, flora melífera y aspectos de polinización. Revisión. Rev Mex Cienc Pecu 2022;13(2):525-548. https://doi.org/10.22319/rmcp.v13i2.5960.

2. Murray J. Description of a new species of *Cryptophagus.* Entomol Mon Mag 1867;4:212-213.

3. Neumann P, Ellis JD. The small hive beetle (*Aethina tumida Murray,* Coleoptera: Nitidulidae): distribution, biology and control of an invasive species. J Apic Res 2008;47(3):181-183. https://doi.org/10.1080/00218839.2008.11101453.

4. Neumann P, Pettis JS, Schäfer MO. Quo vadis *Aethina tumida*? Biology and control of small hive beetles. Apidologie 2016;47:427–466. https://doi.org.ezproxy.uv.mx/10.1007/s13592-016-0426-x.

5. Murcia-Morales M, Heinzen H, Parrilla-Vázquez P, Gómez-Ramos MM, Fernández-Alba AR. Presence and distribution of pesticides in apicultural products: A critical appraisal. Trac-Trend Anal Chem 2022;146:116506. https://doi.org/10.1016/j.trac.2021.116506.

6. Urbina SAD, Inca TAR, Carbonero AMP, Palomas JB. Preparación de quitina fúngica a partir de subproductos de hongos comestibles (*Agaricus bisporus*). Pol Con 2020; 5(4):115-140. doi:10.23857/pc.v5i4.1369.

7. Emmert EA, Handelsman J. Biocontrol de enfermedades de las plantas: una perspectiva (Gram-) positiva. FEMS Microbiol Lett 1999;171(1):1-9.

8. Shoda M. Bacterial control of plant diseases. Am J Biosc Bioeng 2000;89(6):515-521. https://doi.org/10.1016/S1389-1723(00)80049-3.

9. Agarwal M, Dheeman S, Dubey RC, Kumar P, Maheshwari DK, Bajpai VK. Differential antagonistic responses of *Bacillus pumilus* MSUA3 against *Rhizoctonia solani* and *Fusarium oxysporum* causing fungal diseases in *Fagopyrum esculentum* Moench. Microbiol Res 2017;205:40-47. https://doi.org/10.1016/j.micres.2017.08.012.

10. Laribi HH, Bouanane A, Drouiche N, Pauss A, Mameri N. Purification, characterization, and molecular cloning of an extracellular chitinase from *Bacillus licheniformis* stain LHH100 isolated from wastewater samples in Algeria. Int J Biol Macromol 2015;72:1117-1128.

11. Xiao L, Liu C, Xie C, Cai J, Liu D, Chen Y. Heterogeneous expression of chitinase gene from *Bacillus licheniformis* MY75 and the characterization of expressed ChiMY. Wei Sheng wu xue bao. Acta Microbiol Sin 2010;50(6):749-754.

12. Kwon JH, Won SJ, Moon JH, Lee U, Park YS, Maung CE, *et al. Bacillus licheniformis* PR2 controls fungal diseases and increases production of jujube fruit under field conditions. Horticulturae 2021;7(3):49. https://doi.org/10.3390/horticulturae7030049.

13. Clavijo A, Cotes AM. Evaluación de la actividad quitinasa en procesos de control biológico de *Rhizoctonia solani* y *Fusarium oxysporum f. sp*. lycopersici en tomate, mediante fitoinvigorización de semillas en presencia de *Trichoderma koningii*. Rev Colomb Biotecnol 1998;1(2):58-66.

14. Micocci, KC, Moreira AC, Sanchez AD, Pettinatti JL, Rocha MC, Dionizio BS, *et al*. Identification, cloning, and characterization of a novel chitinase from leaf-cutting ant Atta sexdens: An enzyme with antifungal and insecticidal activity. BBA-General Subjects 2023;1867(1):130249. https://doi.org/10.1016/j.bbagen.2022.130249.

15. Dikbaş N, Uçar S, Tozlu G, Ozer TO, Kotan R. Bacterial chitinase biochemical properties, immobilization on zinc oxide (ZnO) nanoparticle and its effect on *Sitophilus zeamais* as a potential insecticide. World J Microbiol Biotechnol 2021;37:173. https://doi.org/10.1007/s11274-021-03138-8.

16. Chung C, Suzanne L, Niemela S, Miller R. One – step preparation of competent *Escherichia coli*: Transformation and storage of bacterial cells in the same solution. PNAS 1989;86:2172–2175. doi:10.1101/pdb.prot101212.

17. Ghose TK. Measurement of cellulase activities. Pure Appl Chem; 1987;59:257-268. https://doi.org/10.1351/pac198759020257.

18. Tucuch HJI, Rangel FMA, Casanova LF, Ruíz SE, Utrera QF, Tucuch HCJ. Control alternativo de *Aethina tumida Murray* (Coleoptera: Nitidulidae) con polvos vegetales. Acta Agric Pecu 2020;6:1. https://doi.org/10.30973/aap/ .

19. González GR, Otero CG, Villanueva JJA, Pérez JA, Soto HRM. Toxicidad y repelencia de *Azadirachta indica* contra *Varroa destructor (acari: varroidae).* Agrociencia 2006;40(6):741-751. https://www.redalyc.org/articulo.oa?id=30240606.

20. Chiang MR, Shelomi M. Anatomical changes of the beetle digestive tract during metamorphosis correspond to dietary changes. J Morphol 2023;284: e21575. https://doi.org/10.1002/jmor.21575.

21. Rivera-Berrío JG, Rivera-García JE. Escarabajos: coleópteros. 1st ed. Córdoba, España: Fondo Editorial RED Descartes; 2023.

22. Thamthiankul S, Moar WJ, Miller ME, Panbangred W. Improving the insecticidal activity of *Bacillus thuringiensis* subsp. aizawai against *Spodoptera exigua* by chromosomal expression of a chitinase gene. Appl Microbiol Biotechnol 2004;65(2):183-192. https://doi.org/10.1007/s00253-004-1606-6.

23. Poria V, Rana A, Kumari A, Grewal J, Pranaw K, Singh S. Current perspectives on chitinolytic enzymes and their agro-industrial applications. Biology 2021;10(12):1319. https://doi.org/10.3390/biology10121319.

24. Hussin NA, Ab Majid AH. Termiticidal activity of chitinase enzyme of *Bacillus licheniformis*, a symbiont isolated from the gut of *Globitermes sulphureus* worker. Biocatal Agric Biotechnol 2020;24:101548. https://doi.org/10.1016/j.bcab.2020.101548.

25. Ajuna HB, Lim HI, Moon JH, Won SJ, Choub V, Choi SI, *et al*. The prospect of hydrolytic enzymes from *Bacillus* species in the biological control of pests and diseases in forest and fruit tree production. Int J Mol Sci 2023;24(23):16889. https://doi.org/10.3390/ijms242316889.

26. Nabti EH, Mokrane N, Ghoul M, Manyani H, Dary M, Megias MG. Isolation and characterization of two halophilic *Bacillus* (*B. licheniformis* and *Bacillus sp*) with antifungal activity. J Ecol Health Env 2013;1(1):13-17. http://dx.doi.org/10.12785/jehe/010102.

27. Ibarra J, Del Rincón MC, Galindo E, Patiño M, Serrano L, García R, *et al*. Los microorganismos en el control biológico de insectos y fitopatógenos. Rev Latinoam Microbiol 2006;48(2):113-120.

28. Abulikemu S, Yesilyurt A, Gencer D, Usta M, Nalcacioglu R. Comparison of the potential activities of viral and bacterial chitinases. Egypt J Biol Pest Control 2021;31(1):91. https://doi.org/10.1186/s41938-021-00435-0.

29. Qi Z, Lei B, Xiong M, Li W, Liao Y, Cai D, *et al.* High-level production of chitinase by multi-strategy combination optimization in *Bacillus licheniformis*. World J Microbiol Biotechnol 2024;40:181. https://doi.org/10.1007/s11274-024-03995-z.