Chapter 7

Biopesticide of Neem Obtained by Enzyme-Assisted Extraction: An Alternative to Improve the Pest Control

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Abstract

The indiscriminate use of chemical pesticides to control pests and diseases without technical assistance instead of solving the pest problems has caused environmental damage, agriculture productivity, and human health. Pesticides can remain for several years in the soil, being able to contaminate rivers and lagoons, animals of shepherding and foods. Besides, in recent years, pests have shown an alarmingly resistance over several pesticides. This makes necessary the use of other natural sources of pesticides that could be degraded avoiding the resistance problem. One of the main sources analyzed is the neem (*Azadirachta indica*) due to its complex content of bioactive triterpenoids. However, cellulosic structures of cell wall conditioned the extraction of these components, acting as physical barrier and avoiding its complete extraction. This chapter included a review of the consequences of the use of chemical pesticides to control pests spread in plant and animals and its repercussions on the environment. Moreover, the advantages of the use of food-grade enzyme preparations as an alternative to elaborate an extract of neem without organic solvents are exposed. The results are promissory and could improve the acaricide and repellent effects of the neem extracts over pests, reducing the negative effect caused by chemical pesticides.

Keywords: contamination, pesticide residues, biopesticides, *Azadirachta indica*, food-grade enzymes

1. Introduction

Pests and diseases have always had repercussions directly on losses of crops and livestock products and indirectly over the income decreases due to insufficient harvests of commercial crops. Chemical pesticides are used in an excessive way and without prior technical assistance to pest distribute control, which instead of solving the problem, has produced strong damage to agricultural and livestock productivity, as well as important environmental effects with implications to public health [1]. Pesticides are chemical substances designed to prevent, delay, repel or fight any pests [2].

In [3], it was mentioned that Mexico ranked fifth in the world in the use of 1,1-bis(4-chlorophenyl)-2,2,2-trichloroethane (DDT) in agricultural programs and fourth place for its use in public health. As many as 69,545 ton of DDT were used in health campaigns for the control of malaria and agricultural activities, from 1957 onward, DDT was applied every 6 months indoors and outdoors with a coverage of 2 g/m^2, and almost 1000 ton DDT/y were used in agricultural areas [4]. Indeed, DDT was used in Mexico until the year 2000, and DDT and its metabolites have been found in the environment [5] as well as in human tissues [5, 6], breast milk [7], raw cow's milk and bovine meat [8, 9]. DDT is a very stable organochlorine pesticide that is almost completely metabolized, but small percentage remains as o,p′-DDT, while the most of its concentration is transformed into p,p′-DDE, which is characterized for its poor solubility in water and high affinity for lipids. It is considered a pollutant of high persistence due to its half-life of up to 15 years in the environment [10]. This pesticide persistence is responsible of the wild flora and fauna deterioration, as well as the contamination of soil, water table, continental, and coastal waters. Besides, pesticides can be incorporate into pasture, vegetables, and edible animals, which when consumed, act as transporters facilitating its accumulation in living organisms [11, 12].

In the state of Chiapas, México, [13] found high levels of total DDT in outdoor soil samples that ranged from 0.002 to 27 mg kg^{-1}, while the levels found by [4] in Chihuahua, México, ranged from 0.001 to 0.788 mg kg^{-1}. Taking into account the guideline for total DDT in residential soil of 0.7 mg kg^{-1} in Canada [14], the soil samples from Chiapas had levels higher than the guideline. The high levels of OCs observed may be due to ongoing usage as well as the emission of old residues from soil. Soils are an important sink and source for persistent organic pollutants to the atmosphere. In many of the cases, the contamination of soil with pesticides is due to its incorrect storage, either by leakage of corroded tanks containing liquid pesticides or by aerial dissemination of powder pesticide. However, when pesticides infiltrate the soil, their dissemination depends on the nature of the pesticide, as well as the composition, moisture, pH, and temperature [12, 15]. Because of that, a small portion of spilled pesticides can generate a high soil contamination. Moreover, soil pesticides infiltration can cause their introduction and distribution in the food chain, accumulating successively on each ecological niche until reaching lethal doses for some constituent organisms of the chain, or until reaching high levels of the trophic network [16].

This problem is aggravated due to the excessive use of pesticides in the agricultural sector, the absence of technological remediation and the lack of safety interval (waiting period) for the harvest of agricultural products, which has significant impacts on public health [17]. Some of the toxic effects of DDT and its metabolite identified in mammalian have been alterations in

fetal development and adverse effects on testicular function (semen, sperm, and sperm motility decrease) due to the mimetization or antagonism of reproductive hormones [18, 19]. Thus, DDT metabolites and DDT are considered endocrine disruptors, with estrogenic properties related to several types of estrogen-dependent cancers, such as breast cancer; therefore, the use of this pesticide was prohibited on the decade of the 1970s in most countries [20]. Nevertheless, pesticides remain in the environment (persistence); therefore, the pesticides have been widely distributed, and their traces can be detected in all areas of the environment (air, water, and soil) [21, 22]; therefore, current tendency is focused on natural sources for biological pesticide control. On the other hand, the indiscriminate and uncontrolled application of synthetic pesticide besides to accumulate residues in the environment and, in some cases, in living beings, has caused resistance in some pest. The first case reported of DDT resistance occurred in 1947, and since then, it has increased alarmingly, and it has been estimated that there are currently around 489 species of pest resistant to 400 different pesticides in the world [23]. The irrational use of synthetic pesticide has produced genotypic and phenotypic changes in many species, generating resistance to the action of most of them, including inorganics, DDT, cyclodienes, organophosphates, carbamates, pyrethroids, juvenile hormone analogues, avermectins, neonicotinoids, and antimicrobial [22–24].

Though the use of pesticides has offered significant economic benefits by enhancing the production and yield of food and fibers and the prevention of vector-borne diseases, evidence suggests that their use has adversely affected the health of human populations and the environment [21]. Because of this problem, several researchers are focused their studies on the identification of new natural sources containing active metabolites that could be used on the control of pest [25, 26].

The development of essential oils (EOs) as plant protection products is especially suited to organic farming as well as to integrated pest management. They are natural in origin and biodegradable, have diverse physiological targets within insects, and, consequently, may delay the evolution of insect resistance [27]. EOs act as fumigants, pesticides, repellents, and antifeeds that could affect some biological parameters such as grown rate, biological cycle, and reproduction [26]. One of the most widely analyzed oils is neem (*Azadirachta indica*) which has a toxic effect over several pests and it is a potential alternative to the synthetic pesticide [28]. Neem oil active compounds are azadirachtin, salannin, nimbin, and their respective analogues; being the azadirachtin the most abundant compound [29]. Azadirachtin acts on the immature stages of the insects avoiding their molt or maturation from larva to pupa and generating mutations in the development of different essential parts for their survival, because it affects their ability to oviposit in mature stage and hatch during the larval stage [30]. Its effect is reinforced by the action from the rest of limonoides, such as salannin and nimbin, which have repellent and antifeeder effects over many insects [31]. Although the concentration of azadirachtin is sufficient and its location well established in the seed, its extraction presents some problems, because it is soluble in polar organic solvents, but slightly soluble in water, besides is photosensitive and thermolabile, which conditions its activity in the oil.

Among the methods used for the extraction of azadirachtin, it could be mentioned the cold extrusion in a mechanical press, maceration, and percolation with the use of organic solvents. Each of the proposed methods stimulates the extraction of azadirachtin but in different proportions. Various researchers have suggested that the high variability in the extraction

of azadirachtin from neem depends on several factors as age of the tree, region of its production, stage of fruit development, availability of the internal portion of the seed, storage conditions of the seed, methods and solvents used for its extraction, and the particle size [32–35]. However, one aspect that has not been taken into account is the physical barrier exerted by the cell wall of the seed that directly affects the availability and extraction of azadirachtin and other acaricidal compounds. This problem has been overcome with great success in the plant extract industry through the application of cellulases or preparations with multiple enzymatic activities (cellulases, hemicellulases, and pectinases) [34, 36, 37].

The assisted extraction by cellulolytic enzymes has proven to be a viable and feasible tool to obtain bioactive metabolites from plants, due to its effect on lignocellulosic structures of its cell wall, which increase the yield of oils, pigments, flavorings, and aromas extracted in comparison with traditional extraction methods [38]. Due to the advantages of the use of cellulolytic enzymes for the production of bioactive metabolites from plants and the inherent need to develop sustainable and environmentally friendly alternatives that avoid the resistance phenomenon, the present study had the purpose of evaluating the use of food-grade enzyme preparation on the hydrolysis of the neem seed to obtain extracts with higher concentrations of azadirachtin, but without the use of solvents.

2. Assisted enzymatic extraction of neem

The study was divided into two stages: (1) determination of optimum activity conditions of four enzyme preparations and (2) evaluation of the azadirachtin release kinetics under optimal conditions of enzyme preparation activities. The enzyme preparations used in this study were Crystalzyme PML-MX, Cellulase 17600, Crystalzyme Cran, and Crystalzyme 100XL, and their optimum pH and temperature conditions were identified by using dehydrated and pulverized neem seed. Then, four volumes (1, 2, 3, and 4 mL) of each enzyme were tested to determine the optimum enzyme concentration to neem seed hydrolysis.

Evaluations of azadirachtin release kinetics were conducted at optimum pH and temperature for each enzyme preparation, and the maximum time required for the azadirachtin extraction was determined.

2.1. Material

Neem seeds were provided by a local producer from Jamapa, Veracruz during June, 2017. One kilogram of 110–120 day old neem seeds (green-yellow coloration) was collected. Harvested seeds were washed and cut in half to extract the cotyledon extraction, which was stored by in freezing at −20°C for 24 h before using.

2.2. Food-grade enzyme preparation cellulolytic activity

Each preparation enzymatic activity was measured by filter paper test [39], where the activity was defined as filter paper units per enzyme milliliter ($FPU{\cdot}mL^{-1}$). After $FPU{\cdot}mL^{-1}$ determination, the effect of the enzyme concentration on the hydrolysis of the neem seed

cellulose structures and the azadirachtin release was evaluated. Neem seed hydrolysis was indirectly determined by the quantification of reducing sugar released during the enzymatic reaction. Total protein was estimated with [40] bovine serum albumin (BSA) as standard, and enzymatic activity (EA) for each enzymatic preparation was defined as changes in absorbance in 0.001 of reducing sugars mg protein^{-1} min^{-1}.

2.3. Optimum conditions of pH, temperature, and enzyme concentration

The optimal conditions of pH and temperature were established based on the enzymatic hydrolysis of the neem seed and the release of azadirachtin. To determine the optimum pH of the enzymatic preparations, 1 g of the dehydrated seed was homogenized with 19 mL of phosphate buffer (3.0, 3.5, 4.0, 4.5, 5.0, and 5.5) and was conditioned at 50°C for 5 min and after 1 mL of the corresponding enzyme preparation was added. The enzymatic reaction was carried out for 2 h and was stopped by immersion in ice water. Subsequently, 1 mL of the sample was taken for reducing sugar determination and 1 mL for azadirachtin quantification by HPLC [41]. Once the optimum pH of each enzyme preparation was identified, the effect of the temperature at 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70°C was evaluated. Additionally, one extract without enzyme added was prepared as control, and the concentrations of reducing sugars released were subtracted from the values obtained in each of the enzymatic treatments. Furthermore, enzyme:neem seed (dry base) ratio was evaluated under optimal conditions of pH and temperature, and for this, 2 g of neem seeds (wet base) were used and 0.5, 1.0, 2.0, and 4 mL of each of the enzyme preparations were tested.

2.4. Kinetics of azadirachtin release

To determine kinetics of azadirachtin release, 100 g of neem seed (wet base) was homogenized with phosphate buffer at the optimum pH previously identify (1:10, w v^{-1}) ratio for 3 min using an Ultra Turray homogenizer, T-25 basic (IKA®, Wilmington, NC). Five extracts were elaborated, one for each enzyme preparation and one without enzyme (control), and were incubated at optimum temperatures for each one. An aliquot was taken at 0, 2, 4, 6, 12, 18, and 24 h for the determination of azadirachtin. All samples were analyzed by triplicate.

2.5. Determination of azadirachtin

Azadirachtin quantification was carried out by the HPLC technique proposed by [41], using a binary HPLC system (Waters 1525) and a photodiode detector (Waters 2996). The analytical separation was carried out with a Nova-Pak C18 column of 4 μm (3.9 × 150 mm) SUM (Waters® Milford, MA). Neem samples were centrifuged and diluted with acetonitrile (1:1, v v^{-1}) before analyzing by HPLC. The samples were filtered through acrodiscs (Millipore) of 0.22 μm, and 20 μl was injected into the column. The flow rate was set at 1 mL min^{-1}, the mobile phase was acetonitrile: water (40:60, v v^{-1}), and azadirachtin was read at 217 nm in a retention time of 3.1 min.

2.6. Statistical analysis

The optimal conditions of pH, temperature, and the enzyme:substrate ratio were analyzed by means of analysis of variance (ANOVA) at a level of significance of $p < 0.05$, and Tukey's tests

were used to evaluate the differences between means with the statistical program Minitab 17.3. The kinetics of release of reducing sugars and azadirachtin were analyzed by a first-order empirical equation $Y_i = Y_e (1-e^{-kt})$, in which the equilibrium concentrations (Y_e) and its release rate constant (k) were determined, and the results were analyzed by ANOVA ($p < 0.05$) to determine differences between treatments.

3. Results and discussion

3.1. Food-grade enzyme preparation activity

In order to achieve the optimum cellulolytic activity of the enzyme preparation, filter paper units (FPU·mL^{-1}) of each one were first determined. Crystalzyme PML-MX and Cellulase 17600 L showed the highest cellulolytic activities with 6.96 and 5.60 FPU·mL^{-1}, respectively. This result is probably due to the presence of cellulases in these enzymatic preparations, since the determination of the activity is carried out with filter paper, which is formed by cellulose [42], while the enzymatic preparations, Crystalzyme 100XL and Crystalzyme Cran, showed lower activities with 2.65 and 2.38 FPU mL^{-1}, respectively, and do not contain cellulases (**Table 1**). These results were used as reference to define the amount of enzyme needed to hydrolyze the neem seed cellulolytic structures and extract the highest concentration of azadirachtin.

3.2. pH and temperature determinations

Determination of the optimal reaction conditions was carried out at 50°C, taking into account that temperature is indicated by the enzyme preparation supplier as the optimum. However, in the evaluations, dehydrated neem seed was used instead of filter paper or crystalline cellulose. **Figure 1** shows the determinations of the optimum pH of each enzyme preparations, and the results indicate that all enzyme preparations perform the hydrolysis of the neem seed under very similar pH conditions regardless of the combinations of cellulolytic activity they contain. Optimal pH of the enzymatic preparations was 5.0 for Crystalzyme Cran and 4.5 for Crystalzyme PML-MX, Cellulase and Crystalzyme 100XL. Of the four enzyme preparations, Crystalzyme PML-MX exhibited the highest hydrolysis of the neem seed with 2.2114 (± 0.1879) mg reducing sugars mL^{-1}

Enzyme preparations	Cellulolytic activity	Activity (FPU mL^{-1})
Crystalzyme PML-MX	Pectinase, endoglucanase, exoglucanase, hemicellulase	6.96
Crystalzyme Cran	Pectinase	2.38
Crystalzyme 100XL	Pectinase and arabinase	2.65
Cellulase 17600	Endoglucanase, exoglucanase, β-glucosidase, pectinase and arabinoxylanase	5.6

Table 1. Enzymatic activities of the food-grade enzyme preparations.

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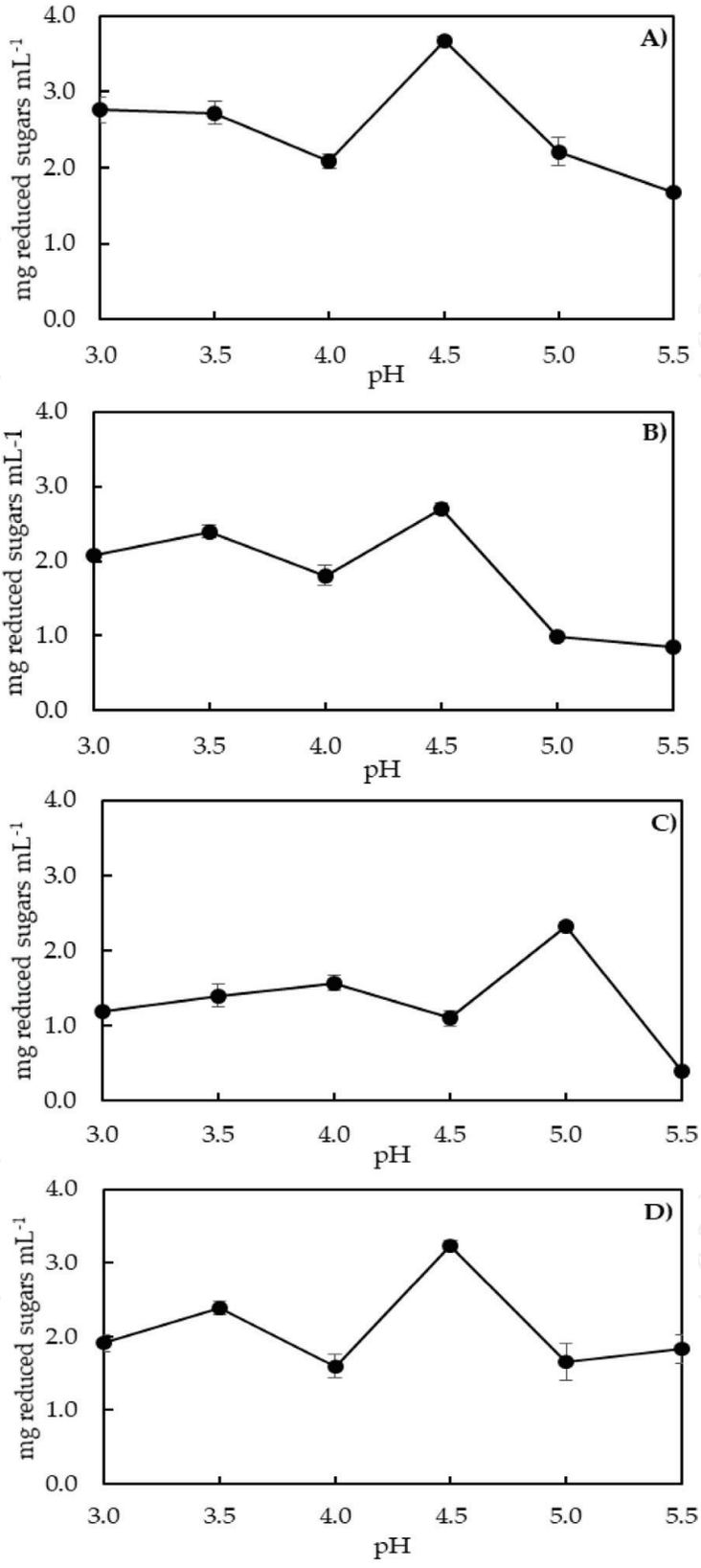

Figure 1. Optimum pH of food-grade enzyme preparations: (A) Crystalzyme PML-MX, (B) Cellulase 17600, (C) Crystalzyme Cran, and (D) Crystalzyme 100XL.

extract. A second reaction was carried out adjusting each hydrolysis to the optimum pH of each enzyme, and the effect of the temperature in the range of 25–70°C was evaluated (**Figure 2**).

Cellulase 17600L, Crystalzyme Cran, and Crystalzyme 100XL shown higher activity at 50°C, while to Crystalzyme PML-MX was at 45°C. These conditions are within the limit considered by [34] to maintain the neem extracts without the loss of azadirachtin. However, it is necessary to evaluate these conditions on the release of azadirachtin, since it is unknown whether the highest hydrolysis of the neem seed ensures maximum concentration of azadirachtin.

3.3. Enzyme concentration

Results of the enzyme concentration effect over hydrolysis of neem seeds could be observed in **Figure 3**. Reducing sugar release was used as indirect measure of neem seed hydrolysis.

Reactions catalyzed by Crystalzyme PML-MX and Cellulase 17600 L presented the highest hydrolysis of the neem seed, probably due to the presence of cellulases in its composition. A total of 1.8072 (± 0.0021), 2.0635 (± 0.0689), 2.0493 (± 0.0521), and 2.0742 (± 0.0283) g L^{-1} reducing sugars were released when 0.5 (3.48 FPU), 1 (6.96 FPU), 2 (13.92 FPU), and 4 mL (27.84 FPU) of Crystalzyme PML-MX were used. On another hand, 1.8034 (± 0.0387), 2.0809 (± 0.0023), 1.9921 (± 0.0456), and 1.9383 (± 0.0781) g L^{-1} reducing sugars were released when 0.5 (2.80 FPU), 1 (5.60 FPU), 2 (11.2 FPU), and 4 mL (22.4 FPU) of Cellulase 17600 were used. These results suggest that an increasing of enzyme concentration in the reaction not necessarily imply higher hydrolysis of the neem seed. Therefore, it is advisable to use the lowest concentration in which the same results were obtained for each enzyme preparation. Crystalzyme Cran and Crystalzyme 100XL showed less hydrolysis of the neem seed after 18 h. Crystalzyme Cran released 1.0022 (± 0.0576) g L^{-1} with the highest concentration of enzyme used (4 mL, 9.52 FPU); however, these results were not statistically different from those found when using 1 (2.38 FPU) and 2 mL (4.76 FPU), since the equilibrium concentration of reducing sugars was 0.9323 (± 0.0786) and 0.9859 (± 0.0341), respectively. On the other hand, the samples hydrolyzed with Crystalzyme 100XL had a maximum reducing sugar release when 4 mL of enzyme (21.2 FPU) was used.

In order to determine the optimum enzyme concentration to neem seed hydrolysis, equilibrium concentration and release rate of the reducing sugars were evaluated. The analysis of the results showed that reducing sugar release rate was not statistically different when 1, 2 or 4 mL for both Crystalzyme PML-MX and Cellulase 17600 L were used. When Crystalzyme Cran was evaluated, the higher rates of release of reducing sugars were obtained with 2 and 4 mL. In contrast, the highest reduced sugar release rate for Crystalzyme 100XL was presented when 1 mL (5.30 FPU) of this enzyme was added. Therefore, based on the analysis of results, it was considered that the units of activity necessary to hydrolyze the neem seed are 6.96, 5.60, 4.76, and 5.3 FPU mL^{-1} for Crystalzyme PML-MX, Cellulase 17600 L, Crystalzyme Cran, and Crystalzyme 100XL, respectively.

After optimum conditions of pH, temperature and enzyme concentration were determined, and the kinetics of azadirachtin released was carried out but using fresh seed instead of dehydrated seed to minimize the risk of losses due to the dehydration process.

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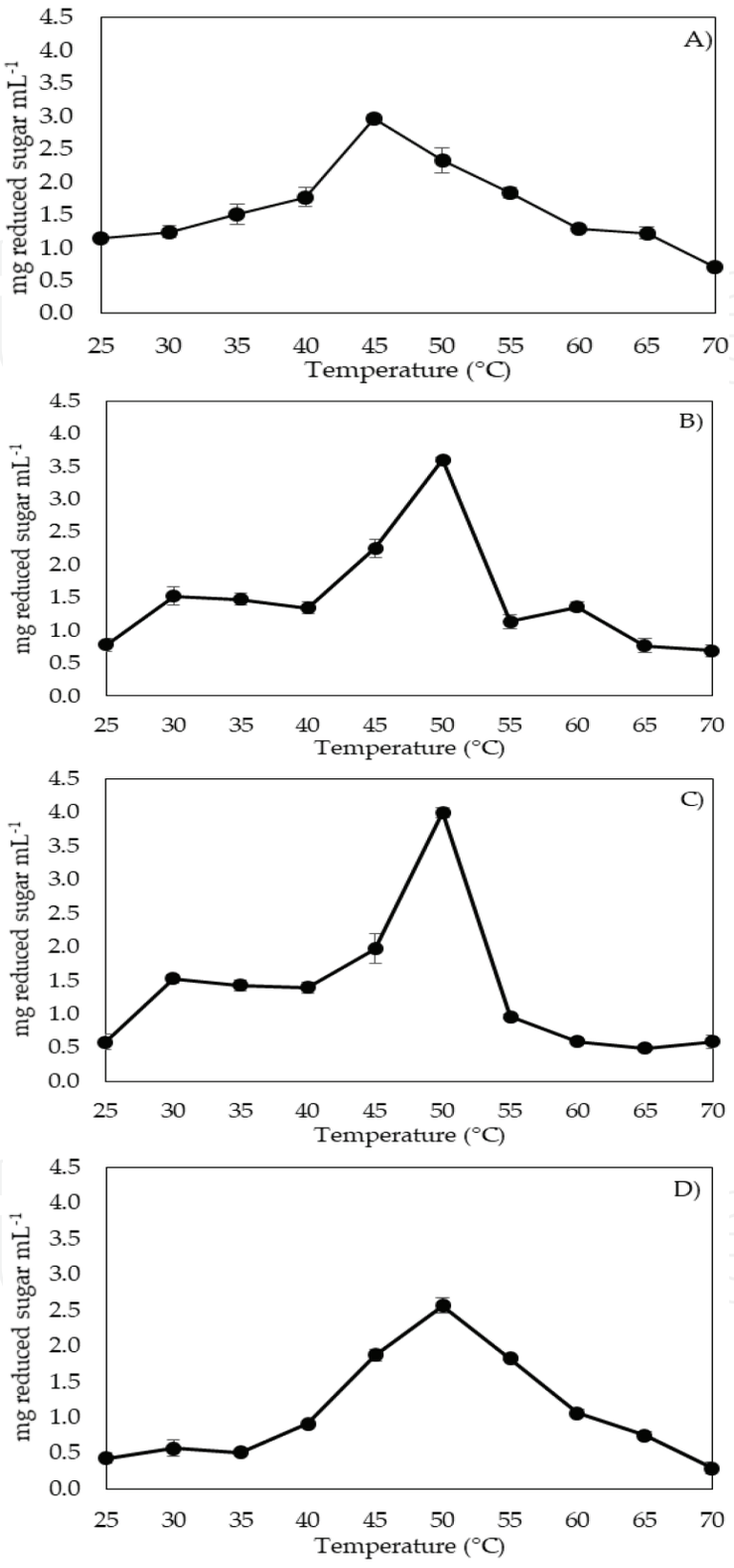

Figure 2. Optimum temperature of food-grade enzyme preparations: (A) Crystalzyme PML-MX, (B) Cellulase 17600, (C) Crystalzyme Cran, and (D) Crystalzyme 100XL.

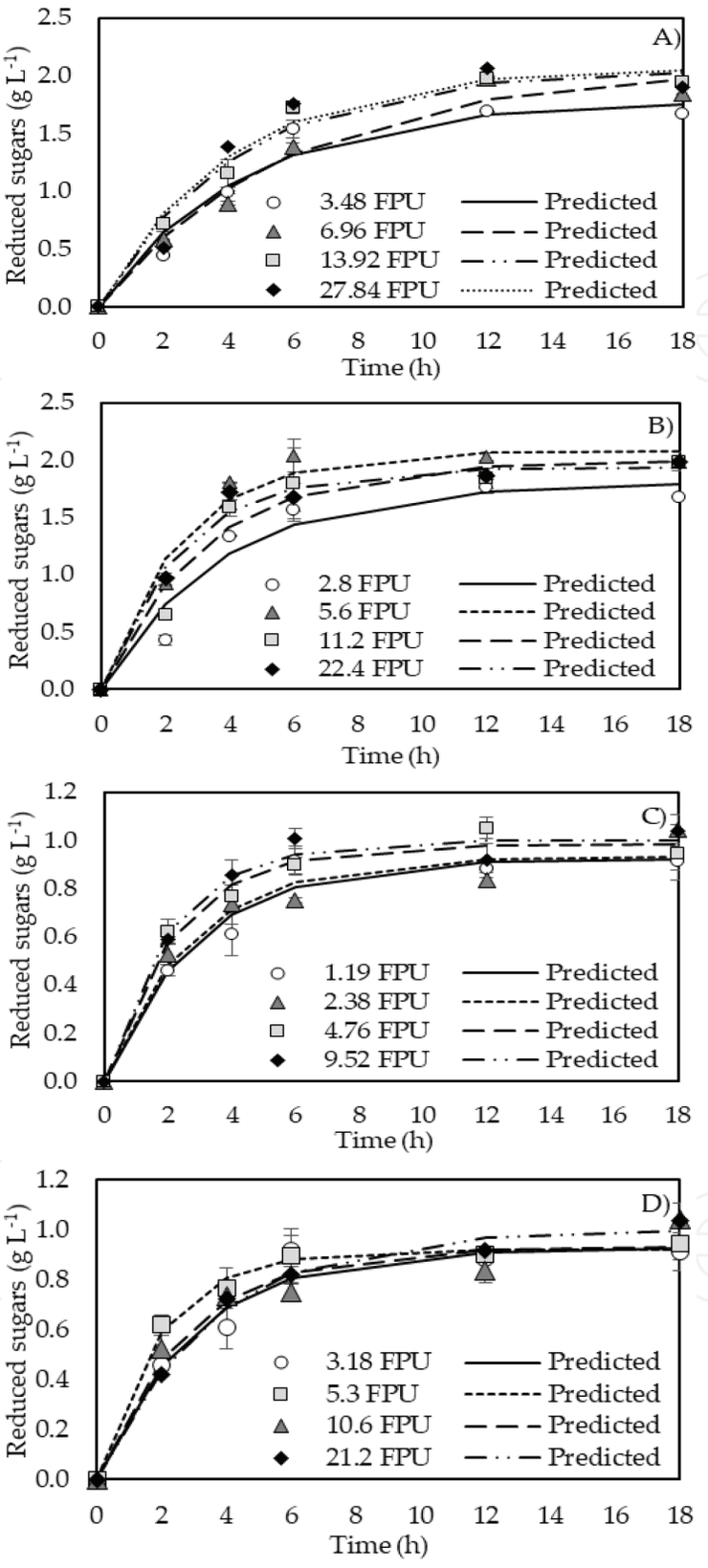

Figure 3. Optimum filter paper unit (FPU) of the food-grade enzyme preparations for hydrolysis of neem seeds. (A) Crystalzyme PML-MX, (B) Cellulase 17600, (C) Crystalzyme Cran, and (D) Crystalzyme 100XL.

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3.4. Azadirachtin release kinetics

Evaluation of the azadirachtin release kinetics was conducted with 100 g of neem seed homogenized and adjusted at 1:10 (w v^{-1}) ratio with phosphate buffer. Enzymatic hydrolysis was conducted under optimal conditions of each enzyme preparation, and azadirachtin were quantified at 0, 2, 4, 6, 12, 18, and 24 h by the HPLC technique, and the results were presented on base of the quantity of neem seed (dry base) used for the neem seed extract elaboration (**Figure 4**). The highest azadirachtin concentrations obtained in this study were 2.55 g kg^{-1} (2550 ppm) and 2.35 g kg^{-1} (2350 ppm) neem seed when Cellulase 17600 L and Crystalzyme PML-MX enzyme preparations were used, respectively. Both of this enzyme preparation include cellulases and pectinases and were statistically different ($p < 0.05$) from the enzymatic preparations, Crystalzyme Cran (pectinases) and Crystalzyme 100XL (pectinases and arabinases).

Azadirachtin concentrations found in this study were higher than those reported by other authors when conventional methods such as extrusion, extraction with solvents (hexane, methanol), and aqueous extraction which reported concentrations of 1080, 565, 400, 150 ppm, respectively [33] and were similar to the obtaining by cold methanol extrusion [32]. However, the concentration obtained is lower than those reported with nonconventional technologies such as extraction with pressurized solvents, which reported concentrations of up to 9510 ppm of azadirachtin [34].

One of the advantages of the use of organic solvents in the extraction process is to improve the solubility of nonpolar components which increase their extraction from vegetable matrices. However, in the present study, only phosphate buffer was used, which could explain the differences of azadirachtin extraction with the pressurized solvent method [34]. Also, in [43], it was reported that in the tropical regions, there are lower concentrations of azadirachtin after extraction processes due to high temperatures, moisture, and storage conditions that could encourage the azadirachtin degradation.

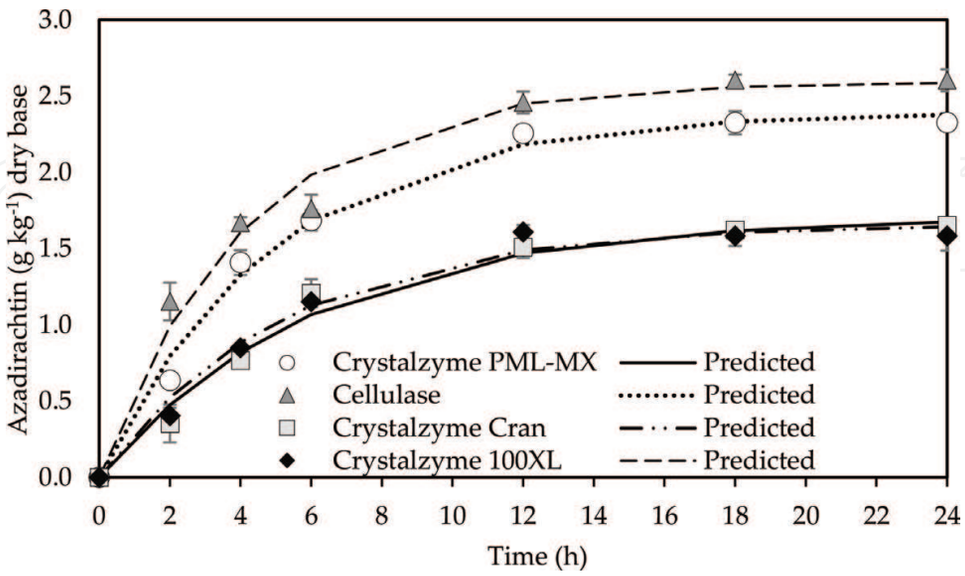

Figure 4. Azadirachtin release kinetics from neem seeds extracted with food-grade enzyme preparations.

Although the highest concentrations of azadirachtin were obtained with the enzymatic preparations, Cellulase 17600L and Crystalzyme PML-MX, also Crystalzyme Cran and Crystalzyme 100XL could be used to elaborate neem extracts because of their azadirachtin concentrations of 1540 and 1690 ppm, respectively, are similar to those contained in commercial acaricidal products elaborated based on neem, but with the use of solvents [32]. This result indicates an advantage and a possible solution to the indiscriminate use of synthetic pesticides and the organic solvents employed in several extractions of active biomolecules. On the other hand, after 18 h of enzymatic hydrolysis, there are no changes on azadirachtin release, which means it is time required to carry out the obtaining of neem seed extracts. In addition, the conditions identified in this study as necessary to neem seed hydrolysis are not extreme or aggressive and could be easily scalable.

4. Conclusions

The present study had the objective of generating an alternative solution to the multiple problems generated by the indiscriminate use of synthetic pesticides. The use of enzymes in the production of metabolites with biological interest has been widely studied in the last decade, due to their specificity and their effect on the cellulose substrate hydrolysis, minimizing the times of obtaining and the necessary costs for their implementation. In addition, its application allows reducing the use of solvents, normally employed in the extraction of neem oil. Besides, the conditions required for neem seed hydrolysis under optimal activity of the enzyme preparations can be easily standardized and scaled for its industrial production. In addition, it was found no affectations on azadirachtin concentration when temperatures of 45 and 50°C were used during enzymatic hydrolysis coinciding with that reported in [34]. However, more studies are needed to determine the stability of azadirachtin under different temperatures and pH conditions during storage.

Although the enzyme-assisted extraction allows the obtaining of neem extracts with higher azadirachtin concentrations than those obtained with conventional methods such as extrusion, cold extrusion, water maceration, and percolation with hexane [32, 33] and in similar concentrations to those obtained by extrusion with cold methanol [32], the amount of neem seeds used in this method was lower, and therefore, the yield was higher than obtained with all these methods. In next studies, components identified as repellents and/or acaricides such as salannin and nimbin should be analyzed. In addition, it is necessary to carry on short-term and long-term *in vitro* and *in vivo* analysis of enzymatic neem extracts on pest with public health impact, defining the vehicle of application and the lethal concentrations for its implementation.

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Conflict of interest

All authors who participate in the elaboration of this manuscript have no conflict of interest to declare.

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