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Formulation process of a biopesticide based on the *Erinnyis ello* betabaculovirus ErelGV: unit operations analysis

Proceso de formulación de un biopesticida a base del betabaculovirus *Erinnyis ello* ErelGV: análisis de las operaciones unitarias

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Abstract

The use of bioproducts developed from active viral ingredients is a promising strategy in integrated pest management. Accordingly, the VG010 betabaculovirus has been identified as an effective agent in controlling the hornworm *Erinnyis ello*. The development of a wettable powder formulation using VG010 as an active ingredient in a biopesticide must have a guaranteed efficacy. An analysis of the unit operations involved in the formulation was carried out using proper operating conditions and production equipment. The active ingredient and finished product characterization revealed the repeatability of variables among batches. The mixing and drying processes were evaluated using operational parameters such as the mixing index and drying kinetics. From this analysis, the most favorable mixing time and drying temperature were selected to obtain a homogeneous product and high insecticidal activity. Viral concentrations, pH of the liquid mixtures, and drying temperature were established as control parameters of the critical points.

Keywords: viral biopesticide, *Erinnyis ello*, formulation process, unit operations.

Resumen

El uso de bioproductos desarrollados a partir de principios activos virales es una estrategia promisora en el manejo integrado de plagas. El betabaculovirus VG010 ha sido identificado como un agente controlador efectivo del gusano cachón *Erinnyis ello*. El desarrollo de una formulación tipo polvo mojable usando VG010 como principio activo en un biopesticida debe garantizar su eficacia. Un análisis de las operaciones unitarias involucradas en el proceso de formulación fue llevado a cabo usando condiciones de operación y equipos apropiados. La caracterización del principio activo y el producto terminado mostró la repetibilidad de las variables medidas en los lotes. Los procesos de mezcla y secado fueron evaluados usando parámetros operacionales como el índice de mezcla y cinéticas de secado. A partir de este análisis, se seleccionó el mejor tiempo de mezcla y la temperatura de secado para obtener un producto homogéneo con alta actividad insecticida. La concentración viral, el pH de las muestras líquidas y la temperatura de secado fueron establecidas como parámetros de control de los puntos críticos.

Palabras clave: biopesticida viral, *Erinnyis ello*, proceso de formulación, operaciones unitarias.

1 Introduction

Providing food for the growing human population with safety standards is a great challenge. Consequently, an urgent need has been identified to provide pest control mechanisms acceptable in the large-scale agricultural sector, which is easily reproducible, environmentally sustainable, and without undesired effects on beneficial organisms (Szewczyk *et al*. 2011; Das *et al*. 2019; Abd-Alla *et al*. 2020). Chemical pesticides have demonstrated negative impacts on the environment and public health. In addition, pest and pathogens have developed resistance to active ingredients.

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Governmental policies are driven to decrease chemical pesticides production to assurance chemical-free foods and promote organic agriculture and integrated pest management programs (Ravensberg, 2011; Mascarin and Jaronski, 2016).

Viral pesticides emerge as a promising alternative to chemical pesticides to obtain safe products for the environment and to human health, compatible with other sustainable agriculture practices and the growing market demand (Szewczyk *et al*., 2011; Popham *et al*., 2016; Grzywacz and Moore, 2017; Abd-Alla *et al*., 2020). Virus and entomopathogenic fungus are widely used as biocontrol agents in several crops with agricultural and economical importance (Rodriguez-Gómez *et al*., 2020). However, it is necessary to establish standardized and economically appropriate protocols for operational processes and the definition of quality control standards for viral biopesticides to ensure compliance with consumer requirements and facilitate its positioning in the market. Furthermore, these protocols would provide the development of adequate industrial production, in which case production plants or facilities should have a proper design and an acceptable production volume to overcome operation costs (Abd-Alla *et al*., 2020; Behle and Birthisel, 2013; Grzywacz and Moore, 2017; Jenkins and Grzywacz, 2000; Szewczyk *et al*., 2011). The production processes for viral bioproducts vary depending on the viral strain, the type of formulation, and the finished product specifications, meaning that a single methodology cannot be used for all preparations (Jenkins and Grzywacz, 2000; Roldão *et al*., 2011). For industrial purposes, monitoring and follow-up procedures for unit operations need to be implemented, as well as the use of standardized and real-time techniques for quality control throughout the process, to guarantee the standardization of the formulation system (Grzywacz and Moore, 2017).

One of the most limiting pests in different crops in the Americas is the moth *Erinnyis ello* (L.) (Lepidoptera: Sphingidae) (Linnaeus, 1758). The insect larvae are mostly foliage consumers of plants from the Euphorbiaceae family, such as cassava (*Manihot esculenta*) and rubber (*Heveae brasiliensis*) and can cause complete defoliation of trees. *E. ello* has been widely studied in countries such as Brazil and Colombia, and has a high number of natural enemies. Among these enemies, a betabaculovirus *E. ello* granulovirus ErelGV, native strain VG010, has remarkable efficacy (Bellotti *et al*., 1992; Carvalho *et al*., 2015). In Colombia, a biopesticide prototype formulated as a wettable powder was developed based on the ErelGV VG010 with mortalities higher than 80% on second and fourth instar larvae (Cuartas *et al*., 2018). Considering that this biopesticide must be applied on the foliage, the formulation was designed to protect the virus from deleterious conditions that could affect its effectiveness, such as environmental factors (i.e., precipitation, relative humidity, UV radiation, and temperature) (Villamizar *et al*., 2010; Camacho *et al*., 2015).

In addition, the formulation should provide stability and efficacy of the active viral ingredient during its production, distribution, and storage, in order to ensure predetermined specifications and mortality under the prescribed conditions for its use (Burges, 1998; Gómez *et al*., 2013; Grzywacz and Moore, 2017). Solid formulations of biologically active ingredients tend to be more stable and could be stored at room temperature. The drying process is a crucial step in the formulation process, however, drying conditions (temperature, relative humidity, exposure time) can affect product efficacy and integrity of the viral capsid (Camacho *et al*., 2013; Chaparro *et al*., 2017). Furthermore, during convective drying, irregular moisture decreasing patterns may affect macroscopic characteristics of the finished product (Santacruz-Vásquez *et al*., 2008). Therefore, this paper reports an analysis of formulation and characterization processes for a biopesticide production based on the ErelGV - VG010, and the design of process control charts to select the critical control points during its production.

2 Materials and methods

The biopesticide formulation process was carried out at a Bioproducts Pilot Plant. Three unique batches of active ingredient were used to produce finished products, and each batch was tested individually. The production conditions are described below.

2.1 Viral active ingredient production

The three batches of active ingredient were produced under the methodology previously standardized by Cuartas *et al*. (Cuartas *et al*., 2018). For viral propagation, the Colombian ErelGV strain codified as VG010 (Barrera Cubillos *et al*., 2014) was multiplied on third instar *E. ello* larvae, that were obtained from an artificially rearing maintained with natural diet under laboratory and greenhouse conditions. The

larvae were incubated under controlled conditions (temperature: 28 ± 4 °C; relative humidity: 75 ± 5%) and fed with cassava leaves (*Manihot esculenta* Crantz), previously sprayed with a virus suspension adjusted to 1×10^6 OB mL^{-1} (occlusion bodies). Dead larvae with symptoms of infection were collected three to seven days after inoculation. To obtain 30 mL of the purified active ingredient, 100 dead larvae were mixed with 30 mL of 0.1% SDS (MolLabs LTDA, Colombia), 40 mL of 96% ethanol (Blamis Dotaciones Laboratorio, Colombia), and then homogenized with a high-efficiency homogenizer at 14000 rpm for 20 min (Ultraturrax T25, IKA®, Germany). To remove larval tissue, the mixture was filtered with sterile gauze and centrifugated at 15000rpm for 20 min (Sorvall® Stratos®, Thermo Fischer Scientific, USA). The volume of active ingredient in each batch was 500 mL. The pellet obtained was resuspended in sterile deionized water and the concentration was adjusted to 1×10^{10} OB mL^{-1}. The viral suspension was frozen at -20 °C until required for formulation (Cool-Lab®, Barnstead Lab- Line, Thermo Fisher Scientific, U.S.A.)

2.1.1 Viral active ingredient characterization

The three batches of viral suspension obtained were characterized by the following physicochemical, microbiological, and insecticidal parameters.

- pH: 450 mL of the viral suspension was placed in a 1 L beaker and kept under constant agitation by a magnetic agitator at 100 rpm (ST15, Torrey Pines Scientific, U.S.A.). The pH was assessed using a potentiometer (C860, Consort®, Belgium).
- Specific weight: a 3 mL sterile syringe was weighed in an analytical balance (AS220, Radwag®, Poland), and filled with the viral suspension. The virus weight was calculated as the difference between empty and filled syringe. Specific weight was determined as the relation among virus weight and sample volume.
- Moisture and solid content: 0.5 g of the viral suspension was placed in a halogen moisture analyzer (MLS 50 - 3, Kern and Sohn GmbH, Germany), and dried at 121 °C to constant weight. The results were reported as percentage of water gram per 100 g of sample.
- Viral concentration: the concentration of viral occlusion bodies was estimated by a spectrophotometric method. Absorbance readings at a wavelength of 280 nm were performed with 1.2 μL of the viral suspension (NanoDrop® DN 1000, Thermo Fisher Scientific, U.S.A.). Results were extrapolated on a previously standardized calibration curve (Barrera *et al*., 2014), calculated with the counts of VG010 OBs stocks by optical microscopy using a Neubauer chamber. The correlation was also supported by bioassay data (correlation coefficient, $R^2 = 0.9389$).
- Total contaminant content: contaminant microbes were assessed by spread plate method. 1 mL of the viral suspension was diluted in 0.1% v/v Tween 80 solution, and decimal dilutions were made serially until 10^{-7}. For bacteria determination, 0.1 mL of 10^{-3}, 10^{-5}, and 10^{-7} dilutions were plated in Nutrient-Agar for bacteria (Oxoid®, Thermo Fischer Scientific, USA), and incubated at 28 ± 0.5 °C for 24 h. For fungi content, 0.1 mL of 10^{-3} and 10^{-5} dilutions were plated in Potato Dextrose-Agar (PDA; Oxoid®, Thermo Fischer Scientific, USA) and incubated at 25 ± 0.5 °C for 72 h. For yeast content, 0.1 mL of 10-1 dilution was plated in Yeast-Malt-Agar (YMA; Oxoid®, Thermo Fischer Scientific, USA) and incubated at 25 ± 0.5 °C for 36 h. Results were expressed as the average number of colony-forming units per gram (CFU g^{-1}) (Quiroga *et al*., 2011).
- Biological activity: 1 mL of viral suspension was diluted in 10 mL distilled water; then decimal dilutions were prepared to adjust the viral concentration to 1×10^5 OB mL^{-1}. A volume of 0.3 mL of viral dilution was sprayed on the adaxial and abaxial surface of cassava leaves (approximately 0.4 g) and dried at room temperature for 30 min. Subsequently, the leaves were placed in a 150 mL plastic container covered with a wet paper towel and a second instar larva of *E. ello* was placed inside. Control treatment consisted of larvae fed on cassava leaves without viral spraying. Each replicate consisted of ten *E. ello* larvae, and there were three replicates per treatment (30 larvae in total). Containers were incubated under controlled conditions for seven days (temperature: 28 ± 2 °C and relative humidity: 70 ± 10%), and larval mortality was evaluated daily. The corrected mortality was calculated using the Schneider-Orelli formula (Zar, 1999).

2.2 *Unit operations of the formulation process*

The unit operations of the production of the three batches (3.5 kg each), are described below, and the adjuvants and their quantities employed are protected by intellectual property as a trade secret and therefore cannot be named in this study.

2.2.1 *Active ingredient suspension with adjuvants*

This process was divided into three stages. In the first stage, a volume of 500 mL of viral suspension was mixed with a first sunscreen protector (UV No.1) employing a high-efficiency homogenizer (Ultraturrax T25, IKA®, Germany) at 6000 rpm for 3 min. The second stage was performed in parallel, which the phosphate buffer pH 8 and the polymer Eudragit S100® (Evonik Industries, Germany) were mixed at 300-350 rpm for 10 min, using a turbine stirrer bar-spin (Orto Alresa®, Spain) with a flat blade (5 cm diameter). Afterward, a pH stabilizer was added to the mixture, and immediately after a non-toxic organic solvent was placed. The mixture was stirred for 2 min under the same conditions. In the third stage, this mixture was added to the suspension obtained in the first stage and mixed using a high-efficiency homogenizer at 6000 rpm for 3 min. The pH of the three mixtures was measured as a control parameter of the operation (C860, Consort®, Belgium).

2.2.2 *Solid-liquid mixing - Mixing index*

An inorganic silicate-based diluent and a second sunscreen protector (UV No. 2) were mixed with a planetary mixer (Ecomax, Hobart®, Germany) and flat beater (33 cm x 22 cm) for 2 min. The stirring conditions were rotational agitation rate 136 rpm and translational rate 60 rpm. Subsequently, the liquid mixture previously obtained was added to the solid mixture, under the same stirring conditions for 12 min.

The mixing index was determined to define the most favorable mixing time required in the solid-liquid mixing operation. For this purpose, three sampling times (3, 6, and 12 min) were defined at three heights inside the mixer: bottom (0-6 cm), middle (6-11 cm), and surface (11-18 cm). For each sampling time and position, a composite sample of 5 g was obtained from three individual samples at the same height. A total of nine composite samples per batch were collected. The concentration of the active ingredient was used as a mixture tracer quantified by q-PCR (Cuartas *et al*. 2018): the sample was diluted 1:20 w/v in sterile ultrapure water, and an aliquot was incubated at 100 °C for 5 min. Subsequently, an amplification procedure was performed using a TaqMan probe and the primers complementary to the granulin gene. The value of the number of copies per gene obtained was interpreted as a direct quantification of OB, considering the simple morphotype of this type of virus (one nucleocapsid per virion).

The mixing index (M) was calculated using Equation 1 proposed by Lacey (Lacey 1954):

$$M = \frac{\sigma_0^2 - \sigma^2}{\sigma_0^2 - \sigma_R^2} \tag{1}$$

where σ^2 is the population variance of the concentrations found at the same time in different sampling positions; σ_0^2 is the maximum variance (upper limit) of a completely segregated mixture calculated according to Equation 2. Finally, σ_R^2 is the minimum variance (lower limit) and corresponds to a fully randomized mixture calculated according to Equation 3.

$$\sigma_0^2 = p(q - p) \tag{2}$$

$$\sigma_R^2 = \frac{p(q - p)}{n} \tag{3}$$

In Equation 2, p is the tracer concentration in the final mixture, and q is the maximum value of the mixture tracer. The letter n in Equation 3 is the number of tracer particles.

2.2.3 *Granulation*

The particle size of the finished product was homogenized using an oscillating granulator (YK160A, Target Pharmatech CO., China) and a mesh opening of 2 mm.

2.2.4 *Forced convection drying - Drying kinetics*

The finished product was dried in a drying chamber with a forced air recirculation system. The granules were distributed homogeneously on stainless steel trays (70 cm x 40 cm), in metal trays, each one with a maximum of 800 ± 5 g and 2-3 mm of thickness. The following drying conditions of temperature and relative humidity were evaluated: (i) 25 ± 2 °C, 48-53%; (ii) 35 ± 2 °C, 27-30%; and (iii) 45 ± 2 °C, 13-17%. The moisture content of the product was determined every 30 or 60 min per triplicate with composite samples of 0.5 g and a halogen moisture

analyzer (MLS 50 - 3, Kern and Sohn GmbH, Germany). The drying process was finished when product moisture content was lower than 5%. The drying rate was calculated with Equation 4, modified from Aral and Beşe (2016).

$$DR = \frac{P_i\left(\frac{MC_i - MC_{i+1}}{100}\right)}{A(t_{i+1} - t_i)} \quad (4)$$

where DR is the drying rate between time t_i and t_{i+1} (kg water h^{-1} m^{-2}); P_i is the viral product weight placed in the trays (kg); MC_i and MC_{i+1} are moisture contents at times t_i and t_{i+1} (%); A corresponds to the superficial area of the trays (m^2).

The data collected were used to plot drying curves (moisture content vs. drying time), and drying rate curves (drying rate vs. drying time). The biological activity was determined to evaluate the effect of the drying conditions on VG010 betabaculovirus insecticidal properties, using the methodology described above with two-time repetitions. Samples of 1 g of finished product from each drying temperature were diluted in distilled water until obtaining 1×10^5 OB mL^{-1} viral concentration. The percentage of efficacy (corrected mortality) was calculated using the Schneider-Orelli formula (Zar, 1999).

2.2.5 *Grinding*

Each finished product batch was ground using a rotor beater mill (SR300, Retsch GmbH, Germany), with a distance rotor and an annular sieve (360 º; 750 μm mesh opening). A feed hopper was used (DR100, Retsch GmbH, Germany), adjusted to 4 cm height from the base, and a speed of 30 vibrations min^{-1}.

2.2.6 *Finished product characterization*

The physicochemical and microbiological characterization of each batch of finished product was performed via the following methods:

- pH: 1 g sample was diluted in 99 mL of distilled and deionized water. The suspension was stirred by a magnetic agitator at 100 rpm (ST15, Torrey Pines Scientific, U.S.A.) and the pH was measured with a potentiometer (C860, Consort®, Belgium).
- Moisture content: methodology described in viral active ingredient characterization.
- Particle size: diameters of 900 particles of formulated product from each batch were determined with a fluorescence inverted microscope (Eclipse TS300, Nikon Instruments Inc., Japan) and an image analyzer (NIS-Elements BR250, Nikon Instruments Inc., Japan).
- Wettability time: 5 g sample was dropped on 200 mL of distilled water. The time for complete wetting was determined (CIPAC, 1989).
- Viral concentration: concentrations were determined by the q-PCR molecular technique previously described, using 1 g samples.
- Total content of contaminant microbes: 1 g of the finished product was diluted until 10^{-7} in 0.1% v/v Tween 80 solution. Bacteria, fungi, and yeast content were assessed as explained before.
- Yield (%): the product recovered from each batch was weighed (PCR, Torrey®, Mexico) and the value was divided among the theoretical weight of the finished product (3.54 kg) to calculate the yield, expressed as a percentage (Camacho *et al*. 2015).

2.3 *Statistical analysis*

A completely randomized experimental design was used in this study with replicated three times under identical conditions (three batches), and three replicates were used for each variable. Biological activity assays used 30 larvae per treatment (10 larvae per replicate and three replicates per treatment). The data were statistically analyzed by estimating normality by the Shapiro-Wilk test, homoscedasticity with Bartlett's test, and variance analysis (ANOVA). Significant differences were determined with Tukey's test (95% confidence) using the software Statistix®8.1 (Analytical Software, U.S.A.).

2.4 *Determination of process acceptance limits*

Four of the response variables quantified in the three production batches were defined as parameters to control critical points: virus concentration of the active ingredient and finished product, pH in the liquid mixture, and final moisture content. Their acceptability limits were identified using Levey Jennings' control charts (Westgard *et al*., 1981; Cooper, 2008). Accordingly, for each critical control

point, a graph was made using average values and standard deviations from the measurements made of nine samples of the batches produced. Each curve in the graph corresponds to the average ± 1, 2, and 3 standard deviations (σ). The UCL and LCL lines (upper and lower control limit, +3σ and -3σ) were described in every control chart; they represent the limits of the variables in relation to the global average between batches.

3 Results and discussion

3.1 Viral active ingredient production

The characterization of the active ingredient and their acceptance limits are summarized in Table 1. Viral suspension pH values were within established limits with a coefficient of variation (CV) among batches of 1.01%, and no significant differences were observed ($F_{2,6}$=2.79, p=0.1394). Similarly, specific weights, moisture content and solid content were comparable in the three batches (CV < 8%), without statistical differences among them (Specific weight: $F_{2,6}$=0.13, p=0.8784; Moisture content: $F_{2,6}$=0.80, p=0.4912; Solid content: $F_{2,6}$=0.82, p=0.4860).

The virus concentrations showed values higher than 1.00×10^{10} OB mL^{-1} by spectrophotometric absorbance, without significant differences related to the three batches (CV=0.46%; $F_{2,6}$=0.41, p=0.6799). The total content of contaminant microbes in the active ingredient corresponded mainly to mesophilic aerobic bacteria ranging between 1×10^{6} and 1×10^{7} CFU mL^{-1}. These values were similar among batches (CV=7.29%; $F_{2,6}$=0.14, p=0.8732), and were 100-folds below defined limits (1×10^{9} CFU mL^{-1}; Grzywacz and Moore, 2017; Jenkins and Grzywacz, 2000). Efficacies of the three batches were equal to or above 80%, without statistical differences found between them (CV=20.28%; $F_{2,6}$=1.98, p=0.2181). The findings were within the required values established by the acceptance limits.

3.1.1 Active ingredient suspension with adjuvants

The pH values through this unit operation are found in Table 2. Compared to the pH of the active ingredient, there was an observed maximum increase of 8%. The values in the first stage ranged from 7.20 to 7.44, in the second stage from 6.99 to 7.22, and the third stage, 7.18 to 7.32. The coefficients of variation in each process stage were 0.70, 1.88, and 1.24%.

3.2 Solid-liquid mixing - Mixing index

The mixing indices calculated were close to the unit, ranging from 0.96 to 1.00 (Figure 1).

Table 1. Characterization of the active viral ingredient used in the production of a biopesticide based on VG010 betabaculovirus (*Erinnyis ello* granulovirus ErelGV).

	Active ingredient			
Characterization parameter	**Batch 1**	**Batch 2**	**Batch 3**	**Acceptance limits**
pH (-)	6.92 ± 0.06	7.02 ± 0.04	7.03 ± 0.04	4 - 9 (Gutiérrez and López-Ferber, 2004)
Specific weight (g mL^{-1})	1.03 ± 0.02	1.04 ± 0.01	1.03 ± 0.01	N.E.
Moisture content	93.49 ± 1.13	92.38 ± 0.73	92.61 ± 0.91	N.E.
Solid content	6.51 ± 1.13	7.62 ± 0.73	7.39 ± 0.91	N.E.
Viral concentration (1010 OB mL^{-1})	13.5 ± 0.7	12.6 ± 1.6	12.5 ± 1.7	1.23 ± 0.13 *
Total content contaminants (107 CFU mL^{-1})	1.07 ± 0.32	0.93 ± 0.20	7.45 ± 2.70	≤100
- Bacteria content (107 CFU mL^{-1})	1.07 ± 0.32	0.93 ± 0.20	7.45 ± 2.70	(Grzywacz and Moore,
- Fungi content (CFU mL^{-1})	<103	<103	<103	2017; Jenkins and
- Yeast content (CFU mL^{-1})	<102	<102	<102	Grzywacz, 2000)
Efficacy (%)	80.0 ± 0.0	80.0 ± 4.1	90.0 ± 8.2	≥80 *

N.E.: acceptable limits were not established.
* Established by the manufacturer.

Table 2. pH values of the active ingredient suspension with adjuvants.

Batch	Stage 1	Stage 2	Stage 3
1	7.26 ± 0.06	7.19 ± 0.04	7.22 ± 0.05
2	7.38 ± 0.07	7.07 ± 0.07	7.25 ± 0.03
3	7.28 ± 0.06	7.10 ± 0.04	7.26 ± 0.05

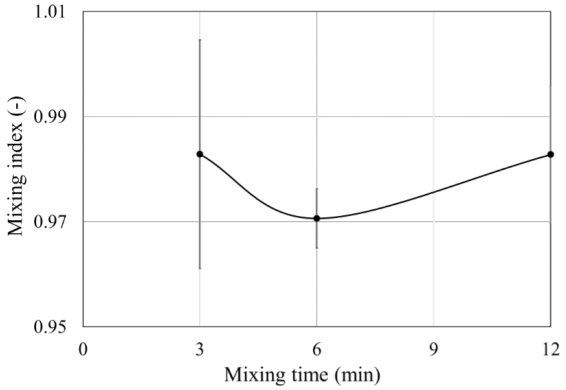

Fig. 1. Mixing index for production of a biopesticide based on VG010 betabaculovirus (*Erinnyis ello* granulovirus ErelGV) using the active ingredient concentration as a mixture tracer.

Furthermore, the standard deviations determined were low compared with the upper limit value (σ_0^2) and corresponded to the variance of a completely segregated mixture. Nonetheless, the points in the graph, where the mixing index is close to 1, were observed after 3 and 12 min of mixing; while an unmixing phenomenon was evident after 6 min. In order to select the most favorable mixing time, the standard deviations of the calculated indices for the batches were considered. The lowest indices were observed in a mixture time of 12 min with the selected tracer.

3.3 Forced convection drying - Drying kinetics

Drying curves for each temperature are shown in Figure 2. The finish time of the drying process was determined to guarantee better microbiological preservation, which is a moisture content below 5% (Roudaut 2008). The divergence over moisture content of the finished product at the three temperatures evaluated could be related to the drying rate and the relative humidity of each operating condition (25 ± 2 °C, 48-53%; 35 ± 2 °C, 27-30%; 45 ± 2 °C, 13-17%).

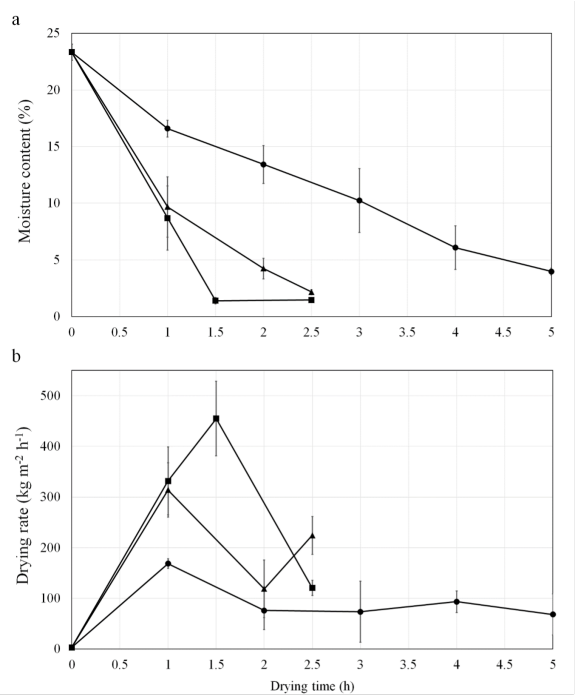

Fig. 2. Drying curves in the production of a biopesticide based on the VG010 betabaculovirus (*Erinnyis ello* granulovirus ErelGV) at different drying temperatures 25 ± 2 °C (●), 35 ± 2 °C (▲), and 45 ± 2 °C (■): a. Moisture content (%); b. Drying rate (kg kg^{-1} h^{-1}).

Analysis of the drying curves revealed different average operating times for the three drying temperatures evaluated: 25 ± 2 °C for 5 h, 35 ± 2 °C for 2.5 h, and 45 ± 2 °C for 2 h. In general, the final humidity percentages ranged from 1 to 4% and did not show significant differences between the batches dried at 25 ± 2°C ($F_{2,6}$=3.68, p=0.0906), while the batches dried at 35 ± 2 °C and 45 ± 2 °C showed statistical differences (35 ± 2 °C: $F_{2,6}$=42.8, p=0.0003; 45 ± 2 °C: $F_{2,6}$=98.6, p<0.0001). At a temperature of 25 ± 2 °C drying process was slower; the rate estimated for the temperatures of 35 ± 2 °C and 45 ± 2 °C demonstrates an acceleration of the superficial water evaporation process (Figure 2b). The mean maximum drying rate for each temperature were: 168.7 ± 9.2 kg m^{-2} h^{-1}, 314.1 ± 53.6 kg m^{-2} h^{-1}, and 454.85 ± 73.6 kg m^{-2} h^{-1}. A proportional relationship between the increase in temperature and the coefficients of variation was evidenced the viral suspension without formulation: 5.5% at 25 ± 2 °C, 17.1% at 35 ± 2 °C, and 20.2% at 45 ± 2 °C.

The mortality of *E. ello* larvae caused by exposure to the three drying temperatures was evaluated. The results show the corrected mortality were 83.33 ± 9.43% (25 ± 2 °C), 56.67 ± 9.42% (35 ± 2 °C), and 71.67 ± 6.87% (45 ± 2 °C), with significant differences between them ($F_{2,15}$=8.00, p=0.0043). Thus, the increase in the drying temperature had a significant negative effect on the biocontrol activity of the VG010 betabaculovirus (*Erinnyis ello* granulovirus ErelGV). The biological activity of the virus observed in the batches dried at 35 ± 2 °C and 45 ± 2 °C was below 80%, and it is considered out of the acceptance limits (Table 1). The drying temperature of 25 ± 2 °C was selected as an adequate drying temperature due to showing the highest biological activity and showed mortalities equal to values observed with the viral suspension without formulation ($F_{1,10}$=0.00, p=0.9999).

3.3.1 Finished product characterization

The quality control of the biopesticide dried at a temperature of 25±2 °C was carried out to guarantee the quality requirements (acceptance limits), and the results are summarized in Table 3. The findings showed that most of physicochemical characteristics did not present significant differences between batches (pH: $F_{2,6}$=2.04, p=0.2110; Moisture content: $F_{2,6}$=2.04, p=0.2110; Particle size: $F_{2,6}$=3.96, p=0.0800), except wettability times ($F_{2,6}$=33.0, p=0.0005). Besides, the results had a low variability determined as the coefficient of variation (pH: CV=0.43%; Moisture content: CV=9.47%; Particle size: CV=4,68%; Wettability time: CV=3.38%). Compared with acceptance limits, these features were within the values established.

The concentrations of batches 2 and 3 were the closest to the minimum acceptable viral concentration in the finished product (Table 3), while the concentration of batch 1 was 1.65% lower. However, high repeatability (CV=4.06%) and no statistical differences were found in the three batches ($F_{2,6}$=2.93, p=0.1297).

The contaminant content of the finished product was entirely due to the presence of bacteria, and the values were below the acceptance limit. The highest concentration of contaminants was found in batch 3, due to the active ingredient used had the highest proportion of contaminants (Tables 1 and 3). The values of microbial contamination were statistically different ($F_{2,6}$=178, p<0.0001), and these concentrations were 100 times lower than the established value and corresponded to 0.16% of the viral concentration. The yields of the biopesticide production were greater than 90% with losses in manufacturing less than 8% (Batch 1: 5.12%; Batch 2: 2.63%; Batch 3: 7.42%; CV = 5.14%).

3.4 Determination of process acceptance limits

The control charts show LCL and UCL estimated for each parameter (Figure 3 and Table 4). For the viral concentration of the active ingredient, the LCL value was 9.28×10^9 OB mL^{-1} and the ULC was 1.76×10^{10} OB mL^{-1} (Figure 3a).

Table 3. Characterization of the finished biopesticide product based on VG010 betabaculovirus (*Erinnyis ello* granulovirus ErelGV).

	Finisheed product batch			
Characterization parameter	**Batch 1**	**Batch 2**	**Batch 3**	**Acceptance limits**
pH (-)	7.12 ± 0.02	7.16 ± 0.01	7.16 ± 0.03	7 - 7.5 *
Moisture content (%)	3.66 ± 0.27	4.04 ± 0.29	4.28 ± 0.05	≤ 10 (Roudaut, 2008)
Particle size (< 20 μm)	92.89 ± 0.79	90.44 ± 1.75	85.55 ± 4.61	N.E.
Wettability time (seg)	24.06 ± 0.09	25.30 ± 0.37	26.14 ± 0.21	≤ 60 seg *
Viral concentration (109 OB g^{-1})	0.71 ± 0.57	1.47 ± 0.14	1.75 ± 0.27	1.0 ± 0.3 *
Total contaminant content (106 CFU g^{-1})	0.98 ± 0.08	1.57 ± 0.01	2.12 ± 0.01	≤ 500 (Grzywacz
- Bacteria content (106 CFU g^{-1})	0.98 ± 0.08	1.57 ± 0.01	2.12 ± 0.01	and Moore, 2017;
- Fungi content (CFU g^{-1})	<103	<103	<103	Jenkins and
- Yeasts content (CFU g^{-1})	<103	<103	<103	Grzywacz, 2000)
Yield (%)	94.88	97.37	92.58	N.E.

N.E.: acceptable limits were not established.
* Established by the manufacturer.

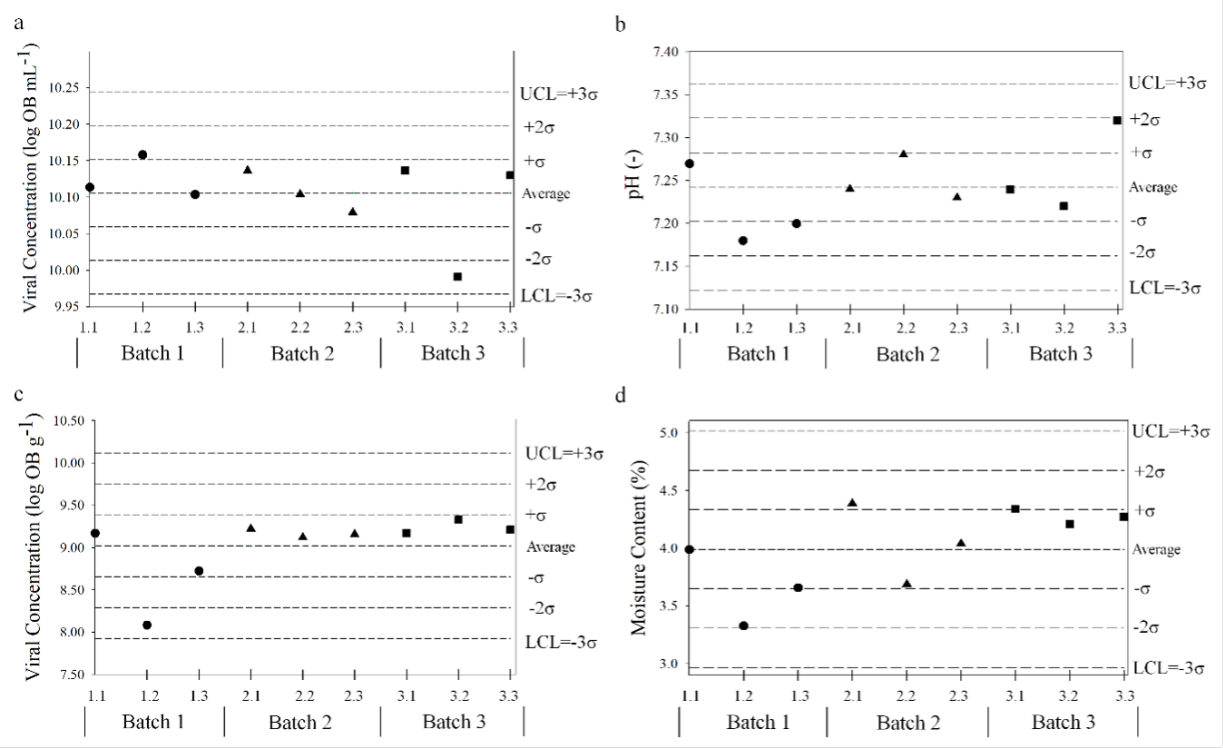

Fig. 3. Process control charts in the production of three batches of a biopesticide based on the VG010 betabaculovirus (*Erinnyis ello* granulovirus ErelGV), Batch 1 (●), Batch 2 (▲), and Batch 3 (■): a. Viral concentration in the active ingredient (OB mL^{-1}); b. pH of the active ingredient suspension with adjuvants (-); c. Viral concentration in the finished product (OB g^{-1}); d. Moisture content of the finished product dried at 25 ± 2 °C (%).

Table 4. Tolerance limits of the critical quality control parameters in the production of a biopesticide based on VG010 betabaculovirus (*Erinnyis ello* granulovirus ErelGV).

Critical point	Critical parameter	LCL	UCL
Viral propagation	Viral concentration	1×10^{10} OB mL^{-1}	1.76×10^{10} OB mL^{-1}
Active ingredient suspension with adjuvants	pH	7.12	7.36
Solid-liquid mixing	Viral concentration	1.00×10^{9} OB g^{-1}	1.31×10^{10} OB g^{-1}
Forced convection drying	Moisture content	2.97%	5.02%

LCL: lower control limit; UCL: upper control limit.

However, to guarantee that the concentration in the finished product is at least 1×10^{9} OB mL^{-1} (Acceptance limit Table 3), the LCL must be equal 1×10^{10} OB mL^{-1} (Table 4).

Control charts analysis reveals that one of the three replicates of viral concentration in batch 3 was not equal to or higher than the minimum expected concentration (1×10^{10} OB mL^{-1}). According to Westgard's rules (Westgard *et al*., 1981), this behavior corresponds to a random error, where a datum is outside the three standard deviations.

The pH control chart was plotted with the data obtained in the mixing operation of the active ingredient suspension and adjuvants (Figure 3b and Table 4). In general, the measured values were within the acceptability limits (LCL and UCL; Table 1), and no systematic or random error was detected according to Westgard's rules (Westgard *et al*., 1981).

Regarding the viral concentration in the finished product, the theoretical LCL value was 8.39×10^{7} OB g^{-1} and the UCL value was 1.31×10^{10} OB g^{-1} (Figure 3c). LCL value was redefined because

the experiments carried out and acceptance limits suggested that it should be above 1×10^9 OB g^{-1} (Table 4). The trend line of this parameter is not center and has a slight upward bias, i.e., most of the experimental concentrations are higher than the average value (Figure 3c).

The lower and upper acceptance limits for moisture content were consistent with the actual production conditions using a drying temperature of 25 ± 2 °C and acceptance limits (Figure 3d and Table 4). All moisture content values were ranged to mean plus or minus two standard deviations ($\pm2\sigma$). Consequently, the wettable powder formulation process for a biopesticide based on the VG010 betabaculovirus (*Erinnyis ello* granulovirus ErelGV) complies with the control conditions specified in these charts and with the established limits for the standardized operating conditions.

4 Discussion

4.1 Viral active ingredient production

The first step to produce high-quality viral products is the characterization of the active ingredient with specific parameters to guarantee an effective quality control system and the reduction of fluctuating performance (Cuartas *et al*., 2018; Gómez *et al*., 2013; Jenkins and Grzywacz, 2000; Ravensberg, 2011). Due to the lack of internationally accepted quality parameters, we established independent acceptance standards based on a conglomeration of other standards from scientific reports of similar products and national regulations. This assessment enabled the establishment of whether the active ingredient is adequate to be used in the biopesticide formulation and to reduce finished product rejection.

The pH value is considered a critical quality control parameter in the formulation process due to its relation with the stability of the occlusion bodies; extreme values cause the release of virions ($4 > pH < 9$) (Abd-Alla *et al*., 2020; Gutiérrez and López-Ferber, 2004; Jenkins and Grzywacz, 2000; Popham *et al*., 2016). On the other hand, the viral suspension specific weight suggests the degree of homogeneity, particularly, the low variation of this characteristic indicates the robustness of the production process. The moisture and solid content results indicate a low number of suspended particles and dissolved solutes in the active ingredient. Peighambardoust *et al*. (2011), highlighted that low values of dry solids and great homogeneity facilitate drying process performance to guarantee small particle size and avoid the thermal inactivation of the biological agent.

The most important parameter evaluated is the viral concentration and could be used to predict whether the final product will fulfil the concentration established by the manufacturers (Table 1). The concentration measurement was performed by an optical method comparing the absorbance values obtained with a calibration curve. Spectrophotometric techniques may present interference in the reading due to the presence of contaminant particles, as result of viral suspension obtention. This method can be replaced by q-PCR quantification due to its higher precision, with or without the presence of non-viral particles (Hitchman *et al*., 2007; Eberle *et al*., 2012).

Considering that the active ingredient (virus) was obtained from larvae that were harvested at environmental temperatures, a high concentration of contaminant bacteria was expected (Cuartas *et al*., 2018). Consequently, the acceptable level for non-pathogen microbial contaminants in the active ingredient must be consistent with the virus production system (Jenkins and Grzywacz, 2000). Currently, the global entities responsible for registering bio-inputs have not yet defined maximally acceptable levels, at least not in their guidelines for applicants. However, some authors have suggested that the total number of contaminating microorganisms in a biopesticide should not exceed 0.1% or 1.0% of the number of infectious propagules (Ravensberg, 2011). For solid products, contaminant concentrations should not exceed 5×10^8 CFU g^{-1} (Jenkins and Grzywacz, 2000). The contaminant content in the active ingredient obtained in the current work was less than 1×10^8 CFU mL^{-1} and guaranteed a low level of contaminants in the final product.

The biological activity of active ingredient efficacy was within the values established by the manufacturer to ensure its performance in field applications. The concentration used in this test (1×10^5 OB mL^{-1}) correspond to lethal concentration 90 (LC90) previously established for this granulovirus (7.7×10^4 OB mL^{-1}) (Cuartas *et al*., 2018). Under the conditions of the viral suspension production, this variable remained stable in the three batches of active ingredient evaluated.

4.2 Unit operations of the formulation process

4.2.1 Active ingredient suspension with adjuvants

In general, the results herein indicated an increase in pH with respect to the viral suspension without excipients. This difference is due to the pH conditions necessary for the dissolution of Eudragit S100® polymer. Nevertheless, the increase in pH is not considered to affect the integrity of viral particles and dissolution in water (Gutiérrez and López-Ferber, 2004; Jenkins and Grzywacz, 2000). The low coefficients of variation determined in the three batches suggested process repeatability and robustness.

4.2.2 Solid-liquid mixing - Mixing index

In this study, the concentration of the active ingredient was used as a mixture tracer to establish the degree of mixing. The quantification methodology used was DNA-based for the active ingredient because it provided virus quantification sensitivity and specificity despite the presence of the formulation excipients (Barrera Cubillos *et al*., 2016). The methodology reported by Lacey (1954) is the most widely used to assess the degree of homogeneity of the mixture using the mixture index. The variance of the data from each sampling time, the completely segregated mixture variance, and the fully randomized mixture variance were used to calculate the mixing index. A value equal to or close to zero corresponds to a completely segregated mixture, and a value of one is obtained when it is fully randomized (Huang and Kuo, 2014; Chou *et al*., 2017). It is crucial to estimate the quantity of the active ingredient in the formulation since a disproportion will hinder the achievement of homogeneity in the mixture. Thus, the mixing index values move away from the unit in a segregate mixture, and there would be less accurate in the product dosage affecting the biocontrol activity in the field (Aulton, 2016). Therefore, a complete randomized mixture guarantees the homogeneity of the viral particles and favors a uniform dispersion and a correct dosage.

4.2.3 Forced convection drying - Drying kinetics

Drying a product is relevant for its preservation and efficacy, as a high moisture content in a product can cause a reduction in the glass transition temperature, and the matrix can change to an elastic state in which the mobility of the molecules and the rate of the chemical reactions is higher. This destabilizes the biological agents and changes the characteristics of the solid prototype, causing denaturation of the genetic material and reduction of its shelf life as a biopesticide. The exposure of the betabaculovirus to high temperatures can be deleterious in some cases due to proteins and the viral genetic material degradation by free radicals obtained from residual molecules of larvae processing (Broeckx *et al*., 2016).

In this work, we observed that the drying temperatures higher than 25 ± 2 °C had a negative effect on biocontrol activity, they were 30% lower than the corrected mortality of the active ingredient. In general, the betabaculoviruses demonstrated tolerance to moderate temperatures (from 40 to 60 °C) for periods between 30 and 180 min, and inactivation at high temperatures, i.e., greater than 60 °C (Behle *et al*., 2006; Camacho *et al*., 2013). The effects of high drying temperatures after long storage periods can be a definitive parameter for establishing the temperature not harmful to a biopesticide. Accordingly, drying may require moderate temperatures for commercial purposes.

The analysis of the drying curves obtained is based on increasing, decreasing, and constant rates periods (Figure 2). Reay and Baker (1985) define the variable rate periods as the removal of moisture from the particles, and the constant rate periods correspond to the stages of internal moisture content removal. Both stages are clearly seen in the drying rate curve of 25 ± 2 °C. The drying rates curves for 35 ± 2 °C and 45 ± 2 °C reveal only the stages of superficial moisture removal because the measurement periods did not show a constant rate state. The initial acceleration of the superficial water evaporation process at 35 ± 2 °C and 45 ± 2 °C was due to the high amount of energy that increases the drying rate compared with 25 ± 2 °C. These initial high drying rates caused a reduction in virus viability measured as corrected mortality and coefficients of variation greater than 10% (Doymaz, 2008; Aral and Beşe, 2016).

For drying rate curves purposes, we used Equation 4, which assumes a water diffusion approach and a Fickian behavior thorough the drying process. Also, this equation represents a macroscopic estimation in a drying process with one single driving force, the gradient of moisture content, and carried out under isothermal conditions (Perré, 2014). Figure 2 shows a first variable rate period that corresponds to an increase of water loss associated with an accelerated diminution of moisture content. The solid surface contains free liquid with a constant vapor pressure at

the surface and equal to the saturated vapor pressure (Perré, 2014). Afterward, the curves described a deceleration time-lapse (Figure 2b), associated with the slope reduction in moisture content curves or constant water content at 45 ± 2 °C (Figure 2a). Falling rate periods are due to moisture diffusion, which may be represented by Fick's second law of diffusion. Furthermore, the solid surface enters the hygroscopic phase, in which the water activity decreases by increasing the saturated vapor pressure and keep a vapor flux (Perré, 2014; Aral and Beşe, 2016). The drying rate curve of 25 ± 2 °C shows a constant rate period since the second hour, linked with variables slopes of moisture content curve; while the drying rate curve of 35 ± 2 °C had the third period associated with superficial water removal.

4.2.4 Finished product characterization

It is essential to select appropriate quality control methods for the type of formulation to ensure the standardization of the production process and guarantee compliance with established requirements. The betabaculovirus-based products show inclusion bodies with high stability and robustness facilitating their processing and formulation (Grzywacz and Moore, 2017). In general, the losses of the finished product through the unit production operations were minimal and were focused on the solid-liquid mixing, granulation, and grinding processes. The pH values obtained were close to neutrality and remaining stable in a range of 4 to 9 (Gutiérrez and López-Ferber, 2004; Popham *et al*., 2016; Abd-Alla *et al*., 2020). For application purposes, this viral-based product will be dissolved in water without the release of the genetic material contained in the virus inclusion bodies, which should be protected by the phosphate buffer incorporated during the formulation process. Furthermore, moisture content of less than 5% enables a long shelf life by reducing contaminant microbe multiplication, which is an important feature to decrease the effect of biotic factors and chemical changes (Roudaut, 2008). In addition, the restriction of humidity, a low wettability time and small particle size prevent the agglomeration of the formulated product and facilitate the dispersion of the biopesticide for its field application (Jenkins and Grzywacz, 2000). The wettability times and particle size demonstrated a positive correlation.

On the other hand, an adequate quantification of the active ingredient in the formulations directly impacts the performance and efficacy of a standard product. Consequently, the OB quantification of the granulovirus proved to be quite challenging due to its small size, requiring observation under phase-contrast microscopy and expert observers that can increase the production cost (Eberle *et al*., 2012). Spectrophotometric techniques are applicable to viral suspensions only before its formulation due to the possibility that adjuvants added in formulation altering light absorption. By contrast, viral quantification techniques based on molecular techniques are robust enough to be applied to multiple commercial productions (Hitchman *et al*., 2007; Wennmann and Jehle, 2014). The current challenges imposed by the development of high-quality biopesticides and the advancement of technologies to reduce costs, the application of these molecular techniques is feasible for the quality control of betabaculovirus-based biopesticides (Barrera Cubillos *et al*., 2016). However, the assessment of the activity by bioassays with insects are essential to estimate OBs infectivity and viability (Grzywacz and Moore, 2017). Due to the *in vivo* production of the viral active ingredient, the presence of microbial contaminants is unavoidable. Therefore, microbial removal can lead to increased production costs (Grzywacz and Moore, 2017; Jenkins and Grzywacz, 2000). Jenkins and Grzywacz suggested a safe and reasonable concentration of non-pathogenic microbial contaminants to 5×10^8 CFU g^{-1} as a limit for dry powder biopesticides. The formulation selected for this biopesticide maintains a moisture content lower than 5% and prevents the proliferation of these contaminants. Good manufacturing practices and aseptic conditions used during the production did not increase the bacterial concentration. A dilution effect of microbial contaminants was observed since most of the adjuvants were sterilized (< 1%).

4.3 Determination of process acceptance limits

The main reasons for not achieving quality standards in viral bioproducts are related to poor production techniques, non-standardized production protocols, and the absence of quality control procedures, affecting the scaling-up processes and continuous production (Grzywacz and Moore, 2017; Jenkins and Grzywacz, 2000). Therefore, in this study, particular attention was given to define critical points, operations, and parameters. Additionally, control charts were used to standardize the production process and provide repeatability between batches. The control of these critical points through biopesticide production

guarantees the established characteristics for the product, including its shelf life and efficacy in the field.

From the experimental results obtained during production at the pilot scale, four critical points were defined: viral propagation, suspension of the active ingredient with adjuvants, solid-liquid mixture, and drying by convection. The parameters selected from each critical point were viral concentration in the active ingredient, pH of the final mixture, viral concentration in the finished product, and final moisture content, respectively. The measurements of the critical parameters during the production of the biopesticide enable manufacturers to monitor, prevent, and correct variations which could reduce the efficacy of the final product. Thereby, the standardization of production will be verified, and each critical point of the process can be monitored to guarantee the quality of the final product.

Conclusions

The findings of this study are the first report of the assessment of unit operations employing proper equipment to produce a biopesticide based on the VG010 betabaculovirus *Erinnyis ello* ErelGV, to guarantee quality acceptance limits. The most favorable operational conditions in each unit operation were selected to assurance the homogeneity of the active ingredient, concentration, and biological activity within the quality standards established. This study is the first step in the scale-up process to increase the batch size, evaluation of new techniques to assess concentration or monitor the process parameters that could be used to guarantee quality standards.

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References

Abd-Alla, A., Meki, I.K. and Demirbas-Uzel, G. (2020). Insect viruses as biocontrol agents: challenges and opportunities. In: *Cottage Industry of Biocontrol Agents and Their Applications*. pp 277-295. Springer Nature, Switzerland. https://doi.org/10.1007/978-3-030-33161-0_9

Aral, S. and Beşe, A.V. (2016). Convective drying of hawthorn fruit (Crataegus spp.): Effect of experimental parameters on drying kinetics, color, shrinkage, and rehydration capacity. *Food Chemistry 210*, 577-584. https://doi.org/10.1016/j.foodchem.2016.04.128

Aulton, M. and Taylor, K. (2016). *Aulton Delineamento de Formas Farmacêuticas*. Elsevier Editora Ltda., Brazil.

Barrera, G.P., Gómez, J., Cuartas, P.E., León, G. and Villamizar, L. (2014). Caracterización morfológica, biológica y genética de un aislamiento Colombiano de granulovirus de *Erinnyis ello* (L.) (*Lepidoptera: Sphingidae*). *Revista Colombiana de Biotecnología 16*, 129-140. https://doi.org/10.15446/rev.colomb.biote.v16n2.41663

Barrera, G.P., Murcia, J., Cerón J.L., Cuartas, P.E., Guzmán, C. and Villamizar, L. (2016). PCR en tiempo real: una metodología útil para la detección y cuantificación de granulovirus. *Revista Colombiana de Biotecnología 18*, 24-31. https://doi.org/10.15446/rev.colomb.biote.v18n2.61514

Behle, R. and Birthisel, T. (2013). Formulations of *Entomopathogens* as Bioinsecticides. In: *Mass Production of Beneficial Organisms: Invertebrates and Entomopathogens*. pp 483-517. Academic Press, U.S.A. https://doi.org/10.1016/B978-0-12-391453-8.00014-5

Behle, R.W., Tamez-Guerra, P. and McGuire, M.R. (2006). Evaluating conditions for producing spray-dried formulations of Anagrapha falcifera nucleopolyhedroviruses (AfMNPV). *Biocontrol Science and Technology 1*, 941-952. https://doi.org/10.1080/09583150600828312

Bellotti, A.C., Arias, V. and Guzman, O. L. (1992). Biological control of the cassava hornworm *Erinnyis ello* (*Lepidoptera: Sphingidae*). *The Florida Entomologist 75*, 506-515. https://doi.org/10.2307/3496132

Broeckx, G., Vandenheuvel, D., Claes, I.J., Lebeer, S. and Kiekens, F. (2016). Drying techniques of probiotic bacteria as an important step towards the development of novel pharmabiotics. *International Journal of Pharmaceutics 505*, 303-318. https://doi.org/10.1016/j.ijpharm.2016.04.002

Burges, H.D. (1998). *Formulation of Microbial Biopesticides*. Kluwer Academic Publishers, Netherlands

Camacho, J., Gómez, M. and Villamizar, L.F. (2015). Microencapsulation of a Colombian *Spodoptera frugiperda* nucleopolyhedrovirus with Eudragit®S100 by spray drying. *Brazilian Archivies of Biology and Technology 58*, 468-476. https://doi.org/10.1590/S1516-8913201500453

Camacho, J., Gómez, M., Villamizar, L.F. (2013). Efecto de la temperatura y de dos procesos de secado sobre la actividad insecticida de un nucleopoliedrovirus de *Spodoptera frugiperda*. *Revista Mexicana de Ingeniería Química 12*, 437-450

Carvalho, R., Ringenberg, R. and Pietrowski, V. (2015). Controle biológico do mandarová da mandioca *Erinnyis ello*. *Embrapa Mandioca e Fruticultura*, Brazil.

Chaparro, M.L., Céspedes, E., Cruz M., Castillo-Saldarriaga, C. and Gómez-Álvarez, M. (2017). Fluidized bed drying of a granulated prototype based on a potential probiotic yeast *Meyerozyma guilliermondii*: Selection of process parameters and drying protectant. *Revista Mexicana de Ingeniería Química 16*, 347-357

Chou, S.H., Song Y. and Hsiau S.S. (2017). A study of the mixing index in solid particles. *KONA Powder and Particle Journal 34*, 275-281. https://doi.org/10.14356/kona.2017018

Cooper, G. (2008). *Basic Lessons in Laboratory Quality Control - QC Workbook*. Bio-Rad Laboratories, Inc., U.S.A.

Cuartas, P.E., Villamizar L.F., Barrera, G.P., Ruíz, J., León-Guillermo, G. and Gómez-Valderrama, J. (2018). Novel biopesticide based on *Erinnyis ello* betabaculovirus: characterization and preliminary field evaluation to control *Erinnyis ello* in rubber plantations. *Pest Management Science 75*, 1391-1399. https://doi.org/10.1002/ps.5259

Das, S., Goswami, A. and Debnath, N. (2019). Application of baculoviruses as biopesticides and the possibilities of nanoparticle mediated delivery. In: *Nano-Biopesticides Today and Future Perspectives*, pp 261-280. Academic Press, India

Doymaz, I. (2008). Convective drying kinetics of strawberry. *Chemical Engineering Processing 47*, 914-919. https://doi.org/10.1016/j.cep.2007.02.003

Eberle, K.E., Wennmann, J.T., Kleespies, R.G. and Jehle, J.A. (2012). Basic techniques in insect virology. In: *Manual of Techniques in Invertebrate Pathology*. pp. 15-74. Academic Press, U.S.A.

Gómez, J., Guevara, J., Cuartas, P., Espinel, C. and Villamizar, L. (2013). Microencapsulated *Spodoptera frugiperda* nucleopolyhedrovirus: insecticidal activity and effect on arthropod populations in maize. *Biocontrol Science and Technology 23*, 829-846. https://doi.org/10.1080/09583157.2013.802288

Grzywacz, D. and Moore, S. (2017). Production, formulation, and bioassay of baculoviruses for pest control. In: *Microbial Control of Insect and Mite Pests: From Theory to Practice*. pp 109-124. Academic Press, U.S.A.

Gutiérrez, S. and López-Ferber, M. (2004). Les stratégies des baculovirus pour franchir l'intestin des insectes. *Virologie 8*, 311-321

Hitchman, R., Siaterli, E., Nixon, C. and King, L. (2007). Quantitative real-time PCR for rapid and accurate titration of recombinant *Baculovirus* particles. *Biotecnhology Bioengineering 96*, 810-814. https://doi.org/10.1002/bit

Huang, A.N. and Kuo, H.P. (2014). Developments in the tools for the investigation of mixing in particulate systems - A review. *Advanced*

Powder Technology 25, 163-173. https://doi.org/10.1016/j.apt.2013.10.007

Jenkins, N.E. and Grzywacz, D. (2000). Quality control of fungal and viral biocontrol agents - Assurance of product performance. *Biocontrol Science and Technology 10*, 753-777. https://doi.org/10.1080/09583150020011717

Lacey, P.M.C. (1954). Developments in the theory of particle mixing. *Journal of Applied Chemistry 4*, 257-268. https://doi.org/10.1002/jctb.5010040504

Mascarin, G.M. and Jaronski, S.T. (2016). The production and uses of Beauveria bassiana as a microbial insecticide. *World Journal of Microbiology and Biotechnology 32*, 177. https://doi.org/10.1007/s11274-016-2131-3

Peighambardoust, S.H., Golshan, T.A. and Hesari, J. (2011). Application of spray drying for preservation of lactic acid starter cultures: A review. Trends *Food Science and Technology 22*, 215-224. https://doi.org/10.1016/j.tifs.2011.01.009

Perré, P. (2014). The proper use of mass diffusion equation in drying modelling: from simple configurations to non-fickian behaviours. *Presentation Hal-01824491*. August. Lyon, France: 19th International Drying Symposium IDS'2014.

Popham, H., Nusawardani, T. and Bonning, B. (2016). Introduction to the use of baculoviruses as biological insecticides. In: *Baculovirus and Insect Cell Expresion Protocols: Methods in Molecular Biology*. pp. 383-392. https://doi:10.1007/978-1-4939-3043-2_19

Quiroga, I., Gómez, M. and Villamizar, L. (2011). Estabilidad de formulaciones a base de granulovirus para controlar Tecia solanivora (Lepidoptera: Gelechiidae) en campo. *Revista Colombiana de Entomología 37*, 27-35.

Ravensberg, W.J. (2011). *A Roadmap to the Successful Development and Commercialization of Microbial Pest Control Products for Control of Arthropods*. Springer, Netherlands

Reay, D. and Baker, C. (1985). Drying. In: *Fluidization*. pp. 529-562. Elsevier, London.

Rodriguez-Gomez, D., Garate-Velez, L., Morales-Piña, A., Gutiérrez-Rojas, M. and Velasco-Alvarez, N. (2020). Increase in the mortality of the entomopathogenic fungus *Metarhizium anisopliae* due to the application of an electric field during conidiation. *Revista Mexicana De Ingeniería Química, 19*, 123-133. https://doi.org/10.24275/rmiq/Bio1601

Roldão, A., Vicente, T., Peixoto, C., Carrondo, M. and Alves, P. (2011). Quality control and analytical methods for baculovirus-based products. *Journal of Invertebrate Pathology 107*, 94-105. https://doi.org/10.1016/j.jip.2011.05.009

Roudaut, G. (2008) Water activity and physical stability. in: water activity, in *Foods: Fundamentals and Applications*. pp 199-213. Blackwell Publishing, U.S.A. https://doi.org/10.1002/9780470376454

Santacruz-Vásquez, V., Santacruz-Vásquez, C., Welti-Chanes, J., Farrera-Rebollo, R., Alamilla-Beltrán, L., Chanona-Pérez, J. and Gutiérrez-López, G. (2008). Effects of air-drying on the shrinkage, surface temperatures and structural features of apples slabs by Means of Fractal Analysis. *Revista Mexicana de Ingeniería Química 7*, 55-63.

Szewczyk, B., Lobo De Souza, M., Batista de Castro, ME, et al (2011) Baculovirus biopesticides. In: *Pesticides - Formulations, Effects, Fate*. pp 25-36. IntechOpen, United Kingdom. https://doi.org/10.5772/13219

Villamizar, L., Barrera, G., Cotes, A.M., Martínez, F. (2010). Eudragit®S100 microparticles containing Spodoptera frugiperda nucleopolyehedrovirus: Physicochemical characterization, photostability and in vitro virus release. *Journal of Microencapsulation 27*, 314-324. https://doi.org/10.3109/02652040903191826

Wennmann, J.T. and Jehle, J.A. (2014). Detection and quantitation of Agrotis baculoviruses in mixed infections. *Journal of Virological Methods 197*, 39-46. https://doi.org/10.1016/j.jviromet.2013.11.010

Westgard, J., Barry, P. and Hunt, M. (1981). A multi-rule Shewhart chart for quality control

in clinical chemistry. *Clinical Chemistry 27*, 493-501. https://doi.org/10.1093/clinchem/27.3.493

Zar, J. (1999). Biostatistical Analysis. Prentice Hall, U.S.A.