



Preparation and Characterization of Oil Nanoemulsion Formulations of *Beauveria bassiana* (Bals.-Criv.)

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Abstract: This study aims to prepare stable thermodynamically dilutable nanoemulsion formulation of *Beauveria bassiana* with the lowest surfactant concentration that could improve its solubility stability. Formulations were prepared from oil in the water nanoemulsion region of phase diagrams subjected to thermodynamic stability tests. We found propanetriol was the highest germination rate at 5% and 10% concentration, 46.66 and 53.33%, respectively. Castor oil achieved a 43.00 germination rate at 1%. Tween 80 gave 54.33 % germination rate at 10%. While Tween 20 showed a 48 % germination rate at 5%. At the concentration, 1% Term 1284 gave 43.33% rate germination. Nanoemulsion composed of propanetriol and nonionic surfactants, with a mean particle size ranging from 25.08 to 75.35 nm, was formulated for various concentrations of the oils and surfactants. Water in oil emulsion was prepared using propanetriol oil, Tween 20, Tween 80, Term 1284, and water. Nanoemulsion of 25.08, 33.75, and 75.35 nm size was obtained at a 45: 15 % ratio of oil and surfactant, and it was found to be stable. The larger droplet size 75.35 nm of formulation Tween 20 and the smaller size was 25.08 nm in the formulation of Term 1284. The higher viscosity value was 16 mPas of formulation Tween 80, and the lowest value was 7.80 in the formulation of Term 1284. To demonstrate the possible employment of these systems, they were used to formulate a nanoformulation pesticide.

Keywords: Formulation, Nanoemulsion, Surfactant, Entomopathogen, *Beauveria bassiana*.

Introduction

There are concerns correlated with the farming use of fungicides, environmental pollution, developed resistance in pests, disagreeable efficacy of delivery, and residues in agricultural products. As such, there is an increasing demand

to look for a new alternative, environmentally friendly product. The purpose of nanotechnology in plant protection is to limit financial losses (Usman *et al.*, 2020). Nanotechnology has been widely used in several

aspects of Agriculture (Abd *et al.*, 2020). Nano bio fungicides describe the subsequent generation of conventional pesticides that would implement more significant benefits such as enhanced efficacy and stability with some active ingredients required (Abd-Elsalam *et al.*, 2019). New strategies for biohybrid nanocide substances could be used as emerging, environmentally friendly antimicrobials against specific pathogenic (Koul 2019). Some eco-friendly fungicides with advanced nanomaterial ingredients have successfully promoted pathogen-killing properties (Abd-Elsalam *et al.*, 2019). The endophytically fungus *B. bassiana* has been introduced successfully and has appeared high potential activity against numerous insect pests (Vega, 2018). The killing mechanism of *B. bassiana* includes adhering to the conidia at the host's body (Aw and Hue 2017). Recently, there is an increased focus on the potential of *B. bassiana* for the biocontrol of plant-insect. Bioinsecticides are promising options for controlling pest populations. Formulation of *B. bassiana* is an alternative option for chemical insecticides in pest management strategies (Dannon *et al.*, 2020). Successful applications of the entomopathogenic fungus *B. bassiana* include developing the proper delivery system as a biocide (Muniz *et al.*, 2020a). Many emulsions can be prepared by the phase titration method. Macroemulsions, Microemulsions, and Nanoemulsions are formed and designed depending on each ingredient's ratio and chemical properties in the ternary phase diagram (Gadhve & Waghmare, 2014). The ternary phase diagram's construction is strongly recommended because it is a beneficial technique to understand the complex interactions series, which can happen when

various ingredients are blended to formulate emulsions. Ternary phase diagrams are applied to know the phase behaviour when the components changing in the ratio of emulsion gradually (Pal *et al.*, 2019). The phase diagram is built by mixing a fixed weight ratio of the emulsion ingredients (Parsi & Salabat 2020). Based on the earlier mentioned background knowledge and to reduce using chemical insecticides, this current study was designed to test the probability of using oil nano formulations of *B. bassiana* as a biocide that could be used for spray applications.

Materials & Methods

Ingredients

Four chemical surfactants, one biosurfactant (BS), and four oils were used as mentioned in table (1), with their supplying source. Conidia of *B. bassiana* was used as an active ingredient. All the experiments were carried in the laboratory condition.

Effect of formulation ingredients on the conidia viability

The effect of formulation ingredients on the *B. bassiana* conidia viability was detected. We followed the procedure previously given by Sandrin *et al.* (2003) to select different compositions.

Three compositions of surfactants (1%, 5%, and 10 %) were used and mixed with 10^8 spores.ml⁻¹ fungal suspension. 200 µl from prepared suspension was sprayed in a petri dish (60 ×15 mm) containing a Ypss medium. The pH was adjusted to 7 ± 1 and incubated at $25 \pm 1^\circ\text{C}$. The assessment of germination was evaluated after 24 h. Conidia were considered germinated successfully when the germ tube's length as long as conidia wide as previously

observed and reviewed by various researchers (Braga *et al.*, 2001; Mwamburi *et al.*, 2015).

Three Petri dishes per one replicate and five replicates for one treatment were used.

Table (1): Ingredients used in the ternary phase diagram study.

No	Compounds	Trade Names	Classes	Supplier/Company
1	Polyoxyl 35 Castor Oil	Termul 1282	Nonionic Surfactant	Emery Olechemicals
2	Polyoxyethylene sorbitan monolaurate	Tween 20	Nonionic Surfactant	DuchefaBiochemie Netherland
3	Sorbitan monooleate	Tween 80	Nonionic Surfactant	Fisher Scientific UK
4	<i>P. aeruginosa</i>	N/A	Biosurfactant	N/A
5	N/A	Corn	Oil	Local Market
6	N/A	Castor	Oil	Local Market
7	N/A	Propanetriol	Oil	Local Market
8	N/A	Safflower	Oil	Local Market
9	Conidia	N/A	Active ingredient	

Influence of oils on conidial viability of *B. bassiana*

The conidia determination is based on oil viability; the germination test of viability was assessed with a similar procedure as described insect of effect of formulation ingredients on the conidia viability.

Oil nanoemulsion formulation preparation

Several parameters have been chosen to prepare *B. bassiana* conidia's oil nanoemulsion, as mentioned below in detail.

Miscibility test pre-formulation

The ingredients' selection was made based on the miscibility among the oil, surfactants, and water to prepare the oil emulsion (Table 2). The quantity of 2 ml of each ingredient mixture was mixed in a 10 ml screw glass tube then vortexed for a proper time to determine the miscibility. The mixture was composed of surfactant, oil, and water. For

that, a similar procedure was repeated eight times. The miscibility standards were based on optical transparency and phase transition of the mixture.

Construction of ternary phase diagram system

The aqueous titration method was used through the ternary phase diagrams technique (Shafiq *et al.*, 2007). The ratios weight to weight was used to mix oil and surfactant. According to the ratio, good volumes of surfactant and carrier were weighed for a total of 0.5 g into a 7 ml screw glass tube.

The mixture was a vortex for a suitable time to obtain an equilibrium solution. The samples were assessed optically for spontaneous emulsification based on clarity, stability, and transparency. The systems' phase behaviour was plotted on phase diagrams using the software Chemix version 3.5 phase diagram plotter (UK).

Table (2): Surfactants, oils, and water grouped in different combinations for phase diagram construction.

Group	Surfactant	Aqueous phase	Oil phase
1	BS	Water	Corn
2	BS	Water	Castor
3	BS	Water	Propanetriol
4	BS	Water	Safflower
8	Tensiofix EW 70	Water	Corn
9	Tensiofix EW 70	Water	Castor
10	Tensiofix EW 70	Water	Propanetriol
11	Tensiofix EW 70	Water	Safflower
15	Termul 1284	Water	Corn
16	Termul 1284	Water	Castor
17	Termul 1284	Water	Propanetriol
18	Termul 1284	Water	Safflower
19	Tween 20	Water	Corn
20	Tween 20	Water	Castor
21	Tween 20	Water	Propanetriol
22	Tween 20	Water	Safflower
23	Tween 80	Water	Corn
24	Tween 80	Water	Castor
25	Tween 80	Water	Propanetriol
26	Tween 80	Water	Safflower

Selection of formulation composition

Different constructed formulations were selected from each phase diagram. One point with the same oil/surfactant ratio was selected based on the phase diagrams' emulsion region. The formulations that showed one phase was chosen. The preferred formulations were subjected to various stability tests.

Characterization of oil nanoemulsion formulation

The characterization of oil nanoemulsion formulation was achieved through the following procedures;

Stability of formulations under centrifugation

The stability of the prepared formulations was measured according to Baboota *et al.* (2007) and Shafiq *et al.* (2007). The selected formulations were centrifuged at 3500 rpm for 30 min. The formulations were stored at room temperature without any phase separation for not less than two days. They were preserved and taken to indicate the phase diagram and processed for further tests.

Stability of oil emulsion formulations under storage conditions

The formulations that passed the centrifuged test were subjected to additional the storage test at

$26 \pm 1^\circ\text{C}$ and $60 \pm 5\%$ RH for three months and two weeks under $54 \pm 1^\circ\text{C}$. The selected formulations were stored according to the agrochemical products vertically prescribed by the Food and Agriculture Organization (FAO) as a standard evaluation to show stability in a tropical climate (Chen *et al.*, 2000; Roland *et al.*, 2003). The observations were made visually to observe any change in the physical appearance of the formulation's samples.

Particle size and Surface tension measurements of emulsion

The particle size of the formulation samples was performed by a Zeta SizerNano-ZS (Malvern, UK) with capillary zeta potential cell (Rodrigues *et al.*, 2014). The emulsion samples surface tension was measured using A tension meter (Tensiometer K6: Model KRUSS, UK). Green (2003) technique was used in this test.

Viscosity measurement of emulsions

The emulsion formulations viscosity was equipped by filling the sample into 20 ml of the viscometer container (Model RheolabQC). First, the viscometer was calibrated. Later, the viscometer's rotating device was inserted in the sample then left to complete the measuring for five minutes. Each run was repeated three times (Roland *et al.*, 2003).

Statistical analysis

Complete Randomized Design (CRD) was used. The data were analyzed using analysis of variance (ANOVA). The highest significant difference (HSD) at 0.05 probabilities was used to separate the means with significant differences through Tukey's standardized range test. All the analysis was done using Statistical Analysis Software version 9.3 (Hatcher & O'Rourke, 2013).

Results

Miscibility Test of the inert ingredients

The miscibility test results of oil, surfactants, and water were grouped into three groups (one phase or transparent and two-phase). The results in table (3) showed that all the surfactants were miscible with propanetriol, and water except BS that was turbid with two phases.

Effect of oils and surfactants on the conidial viability of *B. bassiana*

No significant difference between the surfactants on the germination of conidia of *B. bassiana* in response to the eight surfactants (Termul1282, Tween 20, and Tween 80) was observed. The minimum effect of oils at the concentration of 1% was attained by corn with 11.75% germination, followed by castor and propanetriol with 17.5% and 30.13% germination. Meanwhile, a 5% concentration, castor oil displayed the highest germination rate on the conidia with 28% germination.

The oil of corn and propanetriol give 8.38% and 15.75%, respectively. On the other hand, propanetriol gave a 29.45% germination rate at 10% concentration, followed by castor and corn with germination rates of 19% and 11.50. The minimum effect of surfactants at the concentration of 1% was achieved by Termul 1284 with 42.2% germination, followed by Tween 20 and Tween 80 with 66.5% and 65.7% germination, respectively. Meanwhile, at 5% concentration Term 1284, the conidia displayed the lowest germination rate with 22.7% germination. The surfactant Tween 20 at 10% composition provided the most insufficient effect on conidia with 69.3% and 45% germination for Term 1284 and 42.5 for tween 80.

Table (3): Miscibility test between oils and surfactants used based on spontaneous emulsification.

Surfactant	Canol	Propanetriol	Soybean	Sunflowe	Sesame
	a			r	
Water					
BS	***	***	****	****	***
Termul 1284	***	✓	****	***	***
Tween 20	***	✓	****	****	****
Tween 80	***	✓	****	****	***

*** 2 Phases **** Cream ✓ Transparent with one phase.

Table (4): Conidia germination in different oils and surfactants concentrations.

Germination % / Compositions (%)			
Oils / surfactant	Mean (1%)	Mean (5%)	Mean (10%)
Corn	24.66 b	39.66 b	48.66 a
Castor	43.00 a	44.00 ab	47.00 a
Propanetriol	33.66 ab	46.66 ab	53.33 a
Termul 1284	43.33 a	52.33 a	47.33 a
Tween 20	40.00 ab	48.00 ab	51.00 a
Tween 80	29.66 ab	47.00 ab	54.33 a

L.S.D = 12.34 Means with the same letter are not significantly different

Ternary phase diagram of nanoemulsion system study

The results present three oil /surfactants/water systems were constructed, as shown in Fig. 1 to Fig. 4. Based on these results, all the phase diagrams presented a range of 74% to 80% isotropic region. The phase diagram of the tween 20 and 80 / Propanetriol/water system provided one phase region with 80%. Figs. (3 and 4)

showed the system consists of one phase nanoemulsion in all compositions of surfactant and the oil. In contrast, 74% of the isotropic region was noted in the phase diagram of Term 1284 / Propanetriol/ Water system Fig.2. Fig. 1 presented the ternary phase diagram of pseudomonas/ Propanetriol/ water system turbid with two phases. All these phase diagrams showed one to the two-phase region in the nanoemulsion system.

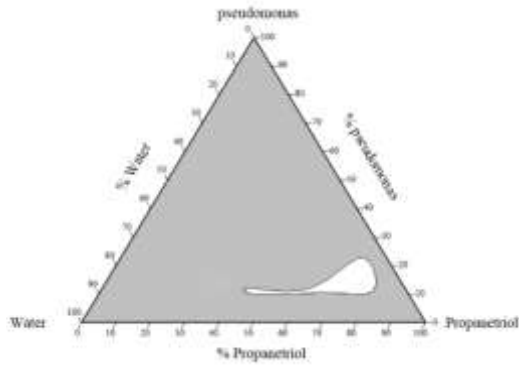


Fig. (1): Phase diagram of pseudomonas / Propanetriol/ Water system showed two-phase.

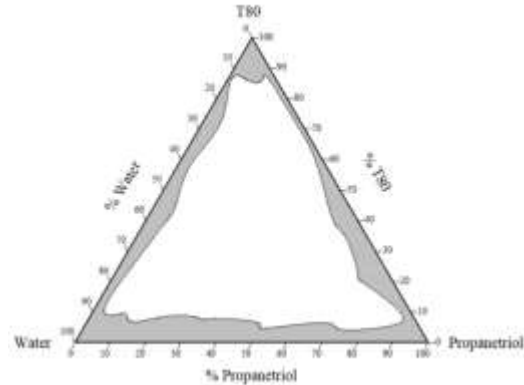


Fig. (4): Phase diagram of Tween 80/ Propanetriol/ Water system showed an 80% one phase region.

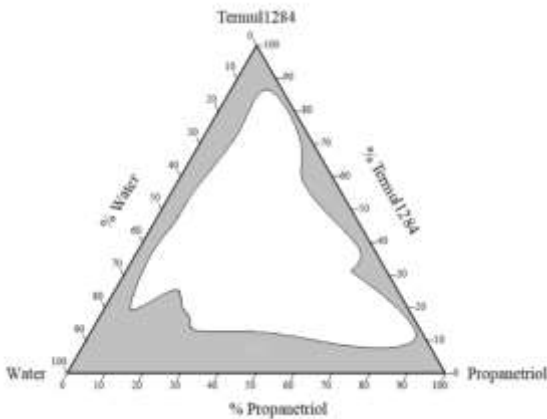


Fig. (2): Phase diagram of Term 1284/ Propanetriol/ Water system 75% one phase.

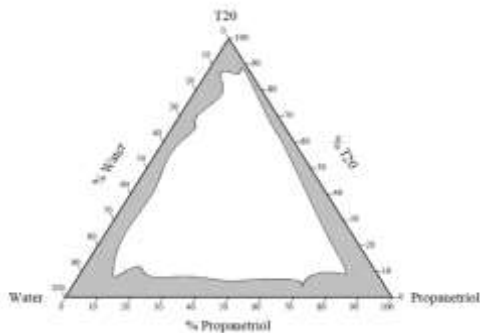


Fig. (3): Phase diagram of Tween 20/ Propanetriol/ Water system showed an 80% one phase region.

Stability of nanoemulsion formulations under centrifuge

The stability of the formulations was characterized after centrifugation to monitor physical stability. The formulations were subjected to a centrifugation force test at $26 \pm 1^\circ\text{C}$. All nanoemulsions prepared displayed recognize stability. There was no phase separation, and the formulations were found to be stable for further thermo test.

Thermostability test of nanoemulsion formulations

Formulations of Term1284, T20 and T80, were observed stable and homogenized because no phase separation was detected under the temperature of $26^\circ\text{C} \pm 1$ and $54^\circ\text{C} \pm 1$ for three months and 14 days storage, respectively (Table 5, Fig. 4). Formulation of ps showed phase separation was detected under the temperature of $26^\circ\text{C} \pm 1$ and $54^\circ\text{C} \pm 1$.

Table (5): Stability test of nanoemulsion formulation with centrifugation at a storage temperature of 26°C and 54°C.

No.	Formulation	Stability under Centrifuge	Stability under 26±1 °C	Stability under 54±1 °C
5	<i>Pseudomonas</i>	no	no	no
6	Term1284	✓	✓	✓
7	T20	✓	✓	✓
8	T80	✓	✓	✓

Particle size and Surface tension measurement of emulsions

The results of table (6) of particle size measurement showed that the smallest particle size was 25.08 nm in the formulation of Term 1284, Followed by 33.75 in the formulation of T80. In contrast, the largest particle size was found in the formulation of T20 with 75.35 nm. Surface tension results for the formulations tested were shown in Table 3.5. That the highest surface tension was 45.05 mN.m⁻¹ in the formulation of Term

1284, Followed by 35.08 mN.m⁻¹ in the formulation of T80. Simultaneously, the lowest surface tension was found in the formulation of T20 with 33.02 mN.m⁻¹.

Viscosity measurements of emulsions

Viscosity measurements presented that all the formulations gave low viscosity; the values ranged from 7.8 to 16 mPas. The formulation of Term1284 showed the lowest viscosity values, with 7.8 mPas, and the highest value obtained by the formulation of T80 with 16 mPas (Table 6).

Table (6): Particle size, Surface tension, and Viscosity values of nanoemulsion.

Formulation	Particle Size(nm)	Std.	Surface Tension(mN.m ⁻¹)	Std.	Viscosity (mPas)	Std.
Term 1284	25.08	0.03	45.05	0.024	7.8	0.223
T20	75.35	0.499	33.35	0.541	14.4	0.303
T80	33.75	0.509	35.08	0.572	16	0.353

Discussion

The present study results showed that the ingredients (oils and surfactants) used to prepare and formulate oil nanoemulsion were nontoxic

for *B. bassiana* conidia. Bouchemal *et al.* (2004) reported that surfactant and oil miscibility could give a primary indicator of the possibility of nanoemulsion formation. The results showed that dilution and solubilization

capability concentrates with water, showed this system is highly proper for effective agrochemical delivery. Many studies stated that reducing the toxicity of nonionic surfactants to a living organism led to increased bio formulation (Jin *et al.*, 2008; Azeem *et al.*, 2009). Silva *et al.* (2005) and Mishra *et al.* (2013) confirmed that the tween 80 and tween 20 (nonionic surfactants) were non-inhibitors to *B. bassiana* growth and they found this surfactant was the best in encouraging the development of *B. bassiana*. It was found the tween 80 and tween 20 were compatible with *B. bassiana* in the germination test (Mwamburi *et al.*, 2015). Azeem *et al.*, (2009) suggested that nonionic surfactants be chosen as less toxic than anionic and cationic surfactants.

Moreover, the results propose that the oils efficiently improved the conidia germination, and it could act as synergists for enhancing the efficiency of *B. bassiana*. The growth in the rate of germinated conidia occurs because the propanetriol was non-inhibitory oil and may improve the tolerance of the conidia under environmental conditions (Muniz *et al.*, 2020b). Ibrahim *et al.* (1999) studied that the conidia of *B. bassiana* formulated in oils frequently germinated over the surface of insect and plant cuticles contrasted with aqueous suspension.

The findings of the ternary phase diagram system presented a large isotropic region. These results may be due to nonionic surfactants' use. The physicochemical properties of surfactants can mix oil with water by decreasing the interfacial tension between water and oil (Soberón-Chávez, 2010; Sharma *et al.*, 2016). On the other hand, the surfactant composition affects the stabilization and droplet size of the emulsion (Bernardi *et al.*, 2011).

Selected points listed in Table 3.2 were formulated with *B. bassiana*. Thus, the best weight ratios of oil and surfactant were determined by their miscibility with water. These results are following Bouchemal *et al.* (2004) and Azeem *et al.* (2009), who stated that the nanoemulsion requires a minimum surfactant ratio of 20% in the formulation of the nanoemulsion.

Stability tests are needed due to their predictive capacity as formulations are subjected to situations designed to promote changes that may happen under market conditions (Ribeiro *et al.*, 2015). It was noted that the centrifugation did not influence phase separation and cracking in all the formulations. Azeem *et al.* (2009) stated that the solubility of the surfactant with oil is a significant factor in preparing nanoemulsion formulations.

Thermodynamic stability presents a long shelf life to the nanoemulsion formulation compared to conventional emulsions (Azeem *et al.*, 2009). Since particle size interferes with coalescence and flocculation determination of particle size is the necessary method to evaluate the colloidal system's stability (Gianeti *et al.*, 2015). The particle size for all formulations remained in the nanoscale range with 200 nm in size that characterizes a nanoemulsion (Forgiarini *et al.*, 2001; Gupta *et al.*, 2016; Tong *et al.*, 2016). Morales *et al.* (2003) found that the nanoemulsion droplet sizes increased by reducing surfactant/water ratios at fixed oil percentage or increasing oil/water percentage at fixed surfactant. Tadros *et al.* (2005) stated that if the particle size is less than 80 nm, it gets superior characteristics compared to conventionally sized emulsions, including optical transparency, high colloidal stability,

and a sizeable interfacial area to volume ratio. Wooster *et al.* (2008) found that the emulsion particle size decreases with the increase of surfactants percentage.

The surface tensions detected were considerably similar for the formulations. The lower surface tension in oil nanoemulsion formulations is advantageous for improving spreading, wetting, and penetrating the oil nanoemulsion (Tadros *et al.*, 2005). The lower surface tension value allows the insecticide droplet particles to penetrate and spread evenly on the leaf surface with smaller contact angles during applications. The decrease in surface tensile value of the formulations in this study describes the reaction among surfactant and oil in reducing the formulations' surface tension.

To manage the formulation decision, perception regarding spray droplet and the propagule of Entomopathogenic interact with the host surface could assist. If particles adhere to the host surface, the droplet must be capable of wetting the host surface. Generally, for a solid to be wet by a liquid, the liquid's surface tension must be lower than the solid's surface energy. Therefore, any prepared formulations must have low surface tension for successful results. Dynamic surface tension is necessary for spray applications. By spraying the droplet-forming process, new droplet surfaces are continually being formed, and the surfactant must disperse to the surface to reduce the surface tension (Jackson *et al.*, 2010). Du *et al.* (2016) reported the wetting, spreading, and penetrating could be increased by the low surface tension of the whole system.

The oil viscosity, the (hydrophilic lipophilic balance) of the surfactant, and miscibility with water represent the critical parameters in

determining the quality of the final nanoemulsion achieved through spontaneous processes emulsification. Similarly, Bouchemal *et al.* (2004) declared that combining the oils and surfactants phase is highly imperative to obtain and characterize the spontaneous nanoemulsion formulations. Furthermore, if the miscibility between surfactants, oil mixture, and water is good, the kinetics of spontaneous emulsification remarkably expressed.

Conclusion

This study was to develop an oil-based nanoemulsion formulation of *B. bassiana* conidia as biological control agents. The nanoemulsion was found to be a practical approach to formulate the conidia of entomopathogenic fungus *B. bassiana*. This work is the first report that conidia of *B. bassiana* prepared as nanoemulsion. It could be an excellent choice to use as a biopesticide. The nanoemulsions were stable after incubation for a long time, up to one year of storage.

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Conflicts of interest

The authors declare that they have no conflict of interests.

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تحضير ووصف مستحضرات زيتية نانوية من *Beauveria bassiana* (Bals.-Criv.)

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المستخلص: هدفت هذه الدراسة إلى تحضير مستحلب نانوي مستقر ديناميكيًا قابل للتخفيف من *Beauveria bassiana* بأقل تركيز من عامل التوتر السطحي والذي يمكن أن يحسن ثبات قابليته للذوبان. حضرت مستحضرات نانوية من الزيت في الماء لمخططات الطور الخاضعة لاختبارات الثبات الديناميكي الحراري. وجدنا أن الكليسيروول كان أعلى معدل تجرثم بتركيز 5% و 10% والتي كانت 46.66 و 53.33% على التوالي. حقق زيت الخروج معدل تجرثم 43.00 عند تركيز 1% Tween80 اعطى معدل تجرثم 54.33% عند تركيز 10%. بينما أظهر Tween20 معدل تجرثم 48% عند تركيز 5%. وعند 1% Term 1284 اعطى 43.33% معدل تجرثم. تمت تحضير مستحلب النانوي المكون من الكليسيروول وعوامل التوتر السطحي غير الأيونية ، بمتوسط حجم الدقائق يتراوح من 25.08 إلى 75.35 نانومتر ، لتركيزات مختلفة من الزيوت وعوامل الخافضة للتوتر السطحي. تم تحضير مستحلب الزيت في الماء باستخدام زيت الكليسيروول ، Tween20 و Tween80 و Term1284 و الماء. تم الحصول على مستحلب نانوي بحجم 25.08 و 33.75 و 75.35 نانومتر بنسبة 45: 15% من الزيت والمواد الخافضة للتوتر السطحي ، ووجد أنها مستقرة. كان حجم القطرة الأكبر 75.35 نانومتر من مستحضر Tween20 والحجم الأصغر 25.08 نانومتر في مستحضر Term1284. كانت قيمة اللزوجة الأعلى 16 ميغا باسكال لمستحضر Tween80 ، وكانت أقل قيمة 7.80 في مستحضر Term1284. لاثبات امكانية استخدام هذه الانظمة ، فقد تم استخدامها في تحضير مبيد افات نانوي

كلمات مفتاحية: مستحضر, مستحلب نانوي, Surfactan, فطريات ممرضة للحشرات , *Beauveria bassiana*.