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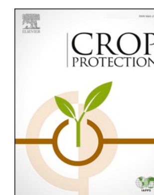

In vitro liquid culture production and post-production pathogenicity of the hybrid *Heterorhabditis bacteriophora* HBH strain

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ABSTRACT

Entomopathogenic nematodes (EPNs) are biological control agents that can be used as alternatives to chemical pesticides against insect pests. Liquid culture is the most suitable method for large-scale production of EPNs. However, to achieve commercial success, the optimization of production parameters is crucial. This study aimed to identify the optimal temperature, pH and dissolved carbon dioxide concentration for the liquid culture production of *Heterorhabditis bacteriophora* hybrid HBH strain. Temperature and pH experiments were conducted in Erlenmeyer flasks, while carbon dioxide experiments were conducted using a 5 L bench-top fermenter. The results revealed that the best yield was achieved at a temperature of 26 °C, a pH of 9, and a dissolved carbon dioxide concentration of 8–9%. The highest yield was observed between the 14th to 16th days in almost all batches. Although no significant difference was found in the pathogenicity of the populations, certain populations obtained from carbon dioxide trials exhibited greater efficacy at lower doses than *in vivo* populations. The liquid production of the HBH hybrid strain is a critical step towards commercial production. Further goals include the optimization of additional parameters for large-scale production. The findings of this study are expected to have practical implications for the industrialization of native hybrid EPN strains.

1. Introduction

Entomopathogenic nematodes (EPNs) have emerged as a promising biological control method for agricultural pests due to their low risk to non-target organisms and environmental safety. EPNs, belonging to the Heterorhabditidae and Steinernematidae families, are soil-dwelling roundworms. They have an obligatory endoparasitic life cycle, which results in the mortality of their insect hosts. (Kaya and Gaugler, 1993). EPNs complete their life cycle inside the host insect except for the infective juvenile (IJ) stage. During the IJ phase, they actively search for hosts in the soil and can survive for an extended period without feeding (Lewis et al., 1992; Hallem et al., 2011). Upon encountering the host in the soil, IJs penetrate through the natural openings and release symbiotic bacteria (*Photorhabdus* spp. for Heterorhabditidae, *Xenorhabdus* spp. for Steinernematidae) into the host haemocoel. The bacteria multiply rapidly and serve as a food source for IJs while secreting a range of secondary metabolites with insecticidal and antimicrobial activities which kill the host in a short period of time. Most hosts have immune responses against infection, such as encapsulation or melanization. However, IJs are able to evade these immune systems and successfully invade the host. Additionally, bacterial symbionts can help suppress the host's immune system (Wang et al., 1994). Following several generations inside the host, the IJs exit the cadaver, move to the soil, and seek out new hosts (Kaya and Gaugler, 1993). EPNs are effective against many soil-borne insects, they can be applied through irrigation systems, and they are suitable for mass production (Peters, 1996, 2013; Shapiro-Ilan et al., 2012; Georgis, 2018). The initial phase of mass production typically involves solid and liquid flask cultures, which are then scaled up in large volume bioreactors. Currently, bioreactor production is considered the most cost-effective method. Thus, they are produced in industrial-scale reactors, mixed with various carriers and salts, and prepared as powdered formulations (Johnigk et al., 2004; Shapiro-Ilan et al., 2004, 2016; Devi, 2018).

However, the usage of EPNs is limited due to certain drawbacks. These include the high initial production expenses, the requirement of technical expertise for their production, their short shelf life, and the necessity for cold chain logistics during transportation (Koppenhöfer et al., 2020; Dunn et al., 2021; Nxitywa and Malan, 2021, 2022; Wang et al., 2022). Consequently, EPN products available in the market are relatively expensive and cannot compete with traditional pesticides. To

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overcome these limitations, extensive research has been conducted to enhance liquid culture optimization, develop novel formulation techniques, and improve the genetics of EPNs (Cruz-Martínez et al., 2017; Devi, 2018; Dunn et al., 2021). For example, early studies on liquid culture investigated the effects of different media content, production parameters, and strain characteristics (Wouts, 1981; Surrey and Davies, 1996; Ehlers, 2001; Shapiro-Ilan et al., 2004). Currently, it is well-established that several factors influence the yield of EPN production. As the production efficiency and yield of IJs increase, costs decrease indirectly. With a focus on production optimization, significant advancements have been made in improving production yield. Another strategy to enhance the effectiveness of EPN products is to commercialize strains possessing superior characteristics through genetic selection. While several local isolates have been identified, the number of isolates with potential for commercialization is limited. To be commercially viable, strains exhibiting desirable traits such as high reproductive capacity, extended longevity, high infectivity, and long-term persistence in the soil are necessary. Through genetic selection, it is feasible to improve important traits and obtain effective EPN strains for commercial use (Ruiz-Vega et al., 2011; Anbesse et al., 2013a, 2013b; Nimkingrat et al., 2013; Sumaya et al., 2018).

As with numerous other organisms, the genetic variability of each strain or isolate results in diverse optimal environmental conditions. Various factors influence the effectiveness and quality of liquid culture production (Dunn et al., 2021). Optimal temperature, pH, viscosity, dissolved oxygen ratio, and agitation rate must be tailored to the specific EPN strain for maximum yield (Ferreira and Malan, 2014; Guadalupe, 2015). In order to achieve maximum yield and quality of EPNs, it is important to optimize the liquid culture production process for each individual strain.

This study focuses on the liquid culture production of the HBH hybrid strain, derived from local isolates of the *Heterorhabditis bacteriophora* species. Although 10 hybrid strains were obtained during the first step, the HBH hybrid strain was determined to have the best characteristics. Compared to the parents, the HBH strain has a high reproductive capacity, high temperature, and drought tolerance (Kongu and Susurluk, 2014; Susurluk et al., 2013). The hybrid strain has been patented based on these characteristics, and its commercial production is expected in the future. Temperature, pH, and dissolved carbon dioxide (CO_2) were selected as production parameters, and their impact on production yield was investigated. The ideal values of these parameters were determined in liquid culture for producing the hybrid strain. After production, quality control trials were conducted, and the pathogenicity of the produced IJs on mealworm larvae was evaluated.

2. Materials and methods

2.1. Studied organisms

The hybrid HBH strain was obtained through the hybridization of *Heterorhabditis bacteriophora* isolates previously isolated from different geographical and climatic regions of Turkey. A crossbreeding procedure was applied to six local *H. bacteriophora* isolates (Sumaya et al., 2018). For the control, one unfertilized female of each strain was transferred into wells without males and incubated for five days.

Production of the initial IJ cultures, and isolation of the eggs and bacteria were performed using the great wax moth *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae). *Tenebrio molitor* Linnaeus, 1758 (Coleoptera: Tenebrionidae) larvae were used to determine the pathogenicity after *in vitro* production. *Galleria mellonella* larvae were reared in the laboratory (Kaya and Stock, 1997), whereas *T. molitor* larvae were purchased from a local distributor.

2.2. Egg isolation and establishment of solid cultures

Monoxenic cultures were prepared according to a modified version of a previously described protocol (Lunau et al., 1993). The last instar of wax moth larvae was inoculated with approximately 100 IJs/larva in a Petri dish and incubated at 24 °C for four days. At the end of the incubation period, the larvae were dissected and approximately 150 fertilized hermaphrodites were collected. Hermaphrodites were transferred to sterile ringer solution and smashed on a vortex using a razor blade, and the eggs were released. After the surfaces of the eggs were cleaned and sterilized, they were incubated in sterile YS medium (5 g Yeast extract, 5 g NaCl, 0.5 g $NH_4H_2PO_4$, 0.5 g K_2HPO_4, 0.2 g $MgSO_4$ * 7 H_2O per 1 l distilled water) for three days and were expected to hatch without contamination. A solution of 0.5 gr NaCl, 1.5 ml 4 mol NaOH and 10 ml sterile water was used for sterilization. The frozen stock bacterial culture (*Photorhabdus luminescens* isolated from hybrid strain) was thawed and inoculated into sterile YS medium in a flask and incubated at 24 °C for 2 days in an orbital shaker. A 200 μl suspension was transferred from the inoculated YS medium to Wouts agar. After the eggs hatched without contamination, they were transferred onto pre-inoculated Wouts agar under aseptic laminar flow chamber. The growth of nematodes on agar was monitored for approximately three weeks and the transition to liquid culture was made when 90% of the population on the agar was IJs.

2.3. Production parameters and liquid cultures

Temperature, pH, and dissolved CO_2 parameters were used for production. The temperature was tested at 24, 26, 28, and 30 °C to determine the optimal temperature that produced the highest yield. Once the best temperature was identified, production was conducted at pH levels of 6, 7, 8, and 9. Finally, the optimal temperature and pH were used to carry out production at dissolved CO_2 concentrations of 6, 7, 8, and 9%.

Temperature experiments were carried out in 250 ml glass Erlenmeyer flasks (Ferreira et al., 2014). To initiate bacterial growth, frozen *P. luminescens* stock cultures were thawed and added to 80 ml of YS medium in flasks. The flasks were then incubated at 24 °C for one day. The bacterial inoculation rate was 4% (v/v), and bovine serum albumin (BSA) medium (composed of 10 g Nutrient broth, 10 g Trypcase soy broth, 5 g Yeast extract, 5 g Peptone, 5 g NaCl, 0.35 g KCl, 0.21 g CaCl2 * 2 H_2O, 3% v/v vegetable oil per 1 L of distilled water) was used for the liquid culture (Kongu and Susurluk, 2014). After one day, 1000 IJs produced in solid media were added to the BSA. The liquid culture production process was initiated at 200 rpm on a heated orbital shaker. To monitor the production process, a 1 ml sample was taken from the liquid medium on the 10th, 12th, 14th, and 16th days, and the number of IJs produced per milliliter was determined. The production process was carried out in five Erlenmeyer flasks for each temperature value, and the experiments were replicated three times in different batches using different cultures.

A specialized benchtop bioreactor (TRL-BIO, TRL Instruments) was utilized to evaluate the effects of pH and CO_2 on nematode production. The bioreactor has flat round base, it has two stage fixed Rushton type impeller blades of 75 mm diameter, and non-porous sparger. Despite the fermenter's maximum working volume of 5 L, 2 L were used in all trials. Ascorbic acid and sodium bicarbonate were used to regulate the pH (if needed), and the peristaltic pumps in the fermenter ensured a constant accuracy of ±0.2 for the pH. For aeration, a consistent 0.5 vvm sterile air was injected into the system throughout the production, however, dissolved O_2 levels was not measured (Strauch and Ehlers, 2000). The CO_2 tests were performed using the CO_2 sensor on the fermenter, which had a sensitivity of 0.05%. The CO_2 provided from a tank and the flow was controlled manually with a rotameter. The flow rate was adjusted according to the CO_2 sensor information to maintain constant dissolved CO_2 ratio (Ehlers, 2001).

To initiate bacterial growth, 4% (v/v) bacteria were inoculated into the growth medium in the fermenters one day before the experiments. The agitation speed was maintained at 250 rpm for all batches, and approximately 25000 IJs were inoculated into the fermenter using the

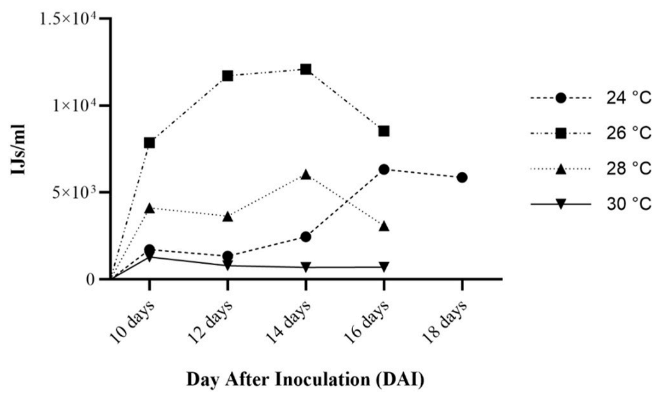

Fig. 1. Infective juvenile yield trends of the productions at different temperatures. The highest number was observed at 26 °C on each DAI.

same ratio as the flask tests. Counts were taken at the end of the 14th day after inoculation (DAI) by using a sampling port on the fermenter. For both the pH and CO_2 experiments, five individual productions were performed for each value, and the tests were carried out with two replicates, resulting in a total of 10 flasks. Fresh batches and cultures were utilized in each replicate.

2.4. Post-production pathogenicity trials

Pathogenicity trials were conducted to control the quality of the IJs produced *in vitro*. The last instar larvae of *T. molitor* were used for the trials (Dunn et al., 2020). One mealworm larva was placed in each well of the cell culture plate and the wells were filled with sterile silver sand at 10% moisture. Six doses (2, 5, 10, 20, 30, and 50 IJs) were applied to each larva, and the plates were sealed with parafilm and incubated at 24 °C for four days (Prasad et al., 2012). We handpicked and applied 2, 5, and 10 IJ doses without water, while 20, 30, and 50 IJ doses were applied with 100 µl of water. At the end of the incubation period, the larvae were dissected, and it was confirmed that the nematodes were responsible for their death. The same experiments were performed using *in vivo* produced IJs (on *G. mellonella* larvae), and the results were compared (Ferreira et al., 2014). Tap water was utilized as the control in both experiments. Each experiment was conducted in three replicates, and 20 mealworm larvae were used for each replicate.

2.5. Data analysis

All data were subjected to the normality and homoscedasticity test prior to statistical analysis. Ordinary one-way ANOVA and Tukey's HSD post-hoc test (0.05) was used to evaluate temperature, pH and dissolve CO_2 reproduction results. Two-way ANOVA, with temperature and DAI as main factors, was used to evaluate interaction effect. Multiple t-tests with Holm-Šídák correction were applied for the *in vivo* – *in vitro*

Fig. 2. Comparison of yields at different temperatures on each day after inoculation (DAI). ** indicates moderate, *** indicates high statistical difference (0.05). Only the comparison of the two highest yields is presented. Error bars represent the standard error mean.

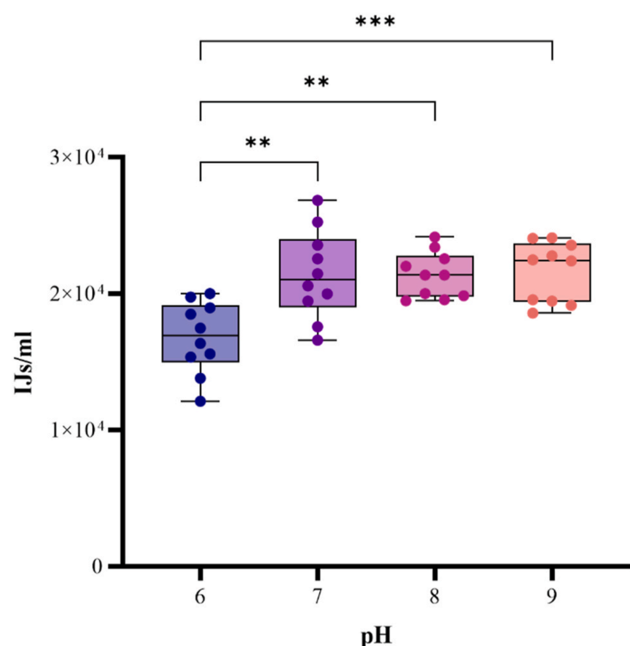

Fig. 3. Comparison of the yield at different pH values. ** indices moderate, *** indicates high statistical difference (<0.05). Bars represent the minimum and maximum values, and the line in the boxes represent mean.

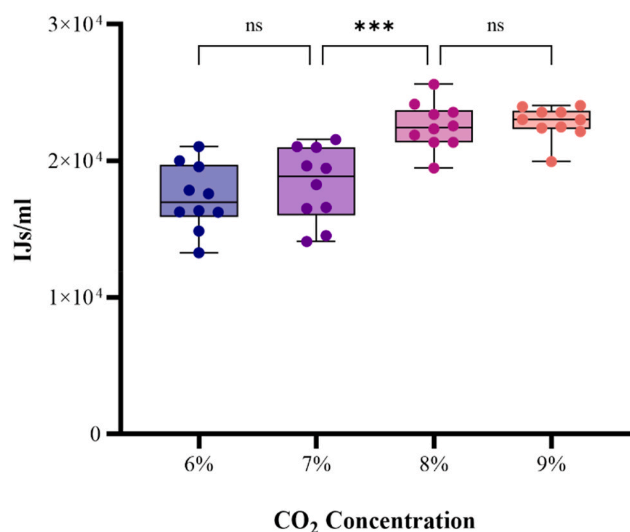

Fig. 4. Comparison of yields at different CO_2 concentrations. *** indicates high statistical difference (0.05). ns indicates there was no significant difference. Bars represent the minimum and maximum values, and the line in the boxes represent mean.

comparison of the pathogenicity results (0.05). A probit analysis for the pathogenicity test results was performed using Minitab v21. All remaining analyzes and graphs were performed using GraphPad Prism v9.

3. Results

3.1. Effect of production parameters on yield

Among all temperature trials, the highest yield was obtained at on the 14th day at 26 °C with 12093 IJs/ml [$F_{(9, 32)} = 4.37$; $P < .001$]. At the end of two weeks, there was a decline in the number of IJs per ml. The counts were terminated after the yield curves showed a downward trend (Fig. 1). Because of the increasing yield in the 24 °C counts, an additional sampling was made on the 18th day. Production at 26 °C always had the highest results from the 10th day when sampling started [$F_{(3, 8)} = 26.8$, $P < .001$ for 10 DAI; $F_{(3, 8)} = 174$, $P < .001$ for 12 DAI; $F_{(3, 8)} = 108$, $P < .001$ for 14 DAI; $F_{(3, 8)} = 70.8$, $P<,001$ for 16 DAI] (Fig. 2). The lowest yield was 693 IJs/ml for 30 °C at the 14th day.

While production at pH 6 in the bioreactor had the lowest yield with 16795 IJs/ml, the results were not significant for all the remaining pH values [$F_{(3, 36)} = 8.75$; $P < .001$]. However, compared to pH 6, the best production was determined to be pH 9, since pH 9 yields had a greater statistical difference than others (Fig. 3). So, the highest yield in pH trials was 21614 IJs/ml.

In the CO_2 trials, the highest IJ production was found at 8% and 9% CO_2 concentrations (22,574 and 22,821 IJs/ml, respectively), and there was no statistical difference between the number of IJs produced at these two values [$F_{(3, 36)} = 18.64$; $P < .001$]. Similarly, there was no statistical difference between the yields at 6% and 7% CO_2 (Fig. 4).

3.2. Post-production pathogenicity

When the pathogenicity of IJs produced at 26 °C and IJs produced *in vivo* were compared, it was observed that the pathogenicity at each dose was statistically similar (0.05). Similar results were observed in the pH experiment, where there were no significant differences in larval mortality between the *in vivo* and *in vitro* populations at any dose (0.05). Although the production yields at pH 7, 8, and 9 were statistically similar, pH 9 was chosen as the optimal condition as it demonstrated a significantly higher yield compared to the lowest yield, and the pathogenicity of IJs produced at this pH was evaluated. However, distinct differences were observed in populations produced at a CO_2 concentration of 9%. At doses of 10, 20, and 30 IJs, the *in vitro* populations exhibited statistically higher pathogenicity compared to those produced *in vivo* [$t_{(4)}$: 6.55, $P = .01$ for 10 IJs; $t_{(4)}$: 6.55, $P < .01$ for 20 IJs; $t_{(4)}$: 6.55, $P < .01$ for 30 IJs] (Fig. 5). As expected, the pathogenicity of the tested infective juvenile populations increased with higher doses, resulting in approximately 70% larval mortality at 20 IJs, and more than 95% at 50 IJs. When assessing the LD_{50} and LD_{90} values, it was found that the *in vitro* produced populations demonstrated lower LD_{90} values in the CO_2 and pH experiments (Table 1), compared to those produced *in vivo*. Similarly, the *in vitro* produced populations in the pH experiments exhibited lower LD_{50} values than those produced *in vivo*.

4. Discussion

The most commonly utilized technique for EPN production is through liquid culture, which demands a high level of technical expertise and is influenced by numerous factors that have a significant impact on the yield (Ehlers, 2001; Cortés-Martínez and Chavarría-Hernández, 2020; Dunn et al., 2021). The optimal production conditions for each strain are different. Therefore, unique production processes are necessary for each strain (Ulu and Susurluk, 2018; Neira-Monsalve et al., 2019; Dunn et al., 2020). The present study revealed that the ideal temperature for producing the HBH hybrid strain of *H. bacteriophora* is 26 °C. The optimal pH was 9 based on the highest statistical difference observed at pH 9 compared to pH 6, which resulted in the lowest yield. In the CO_2 trials, the highest yields were recorded at 8% and 9% concentrations. In almost all trials, the maximum number of IJs was attained on the 14th day. The pathogenicity of all the *in vitro*-produced populations in the study was compared with that of the *in vivo*-produced populations in *G. mellonella* larvae, and mostly no difference in population pathogenicity was observed (Ferreira et al., 2016; Dunn et al., 2020; Kong et al., 2022). However, in some populations from the CO_2 experiments, higher outcomes were seen at lower IJ doses. In summary, the HBH hybrid strain demonstrated the highest yield under the following conditions: 26 °C, pH 9, and CO_2 concentrations of 8–9%.

Since the beginning of the mass production of EPNs, efforts have

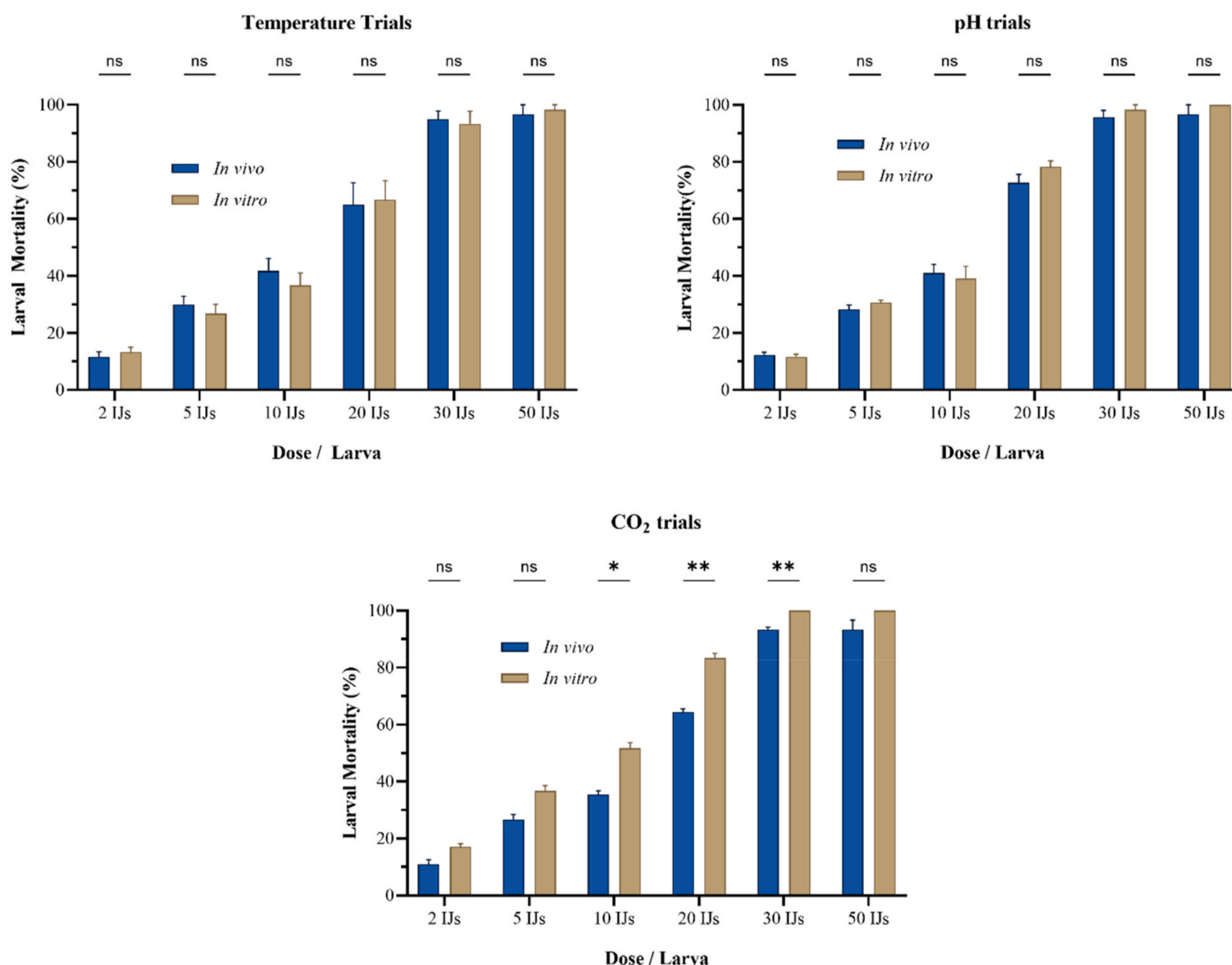

Fig. 5. Comparison of the larval mortality of *in vitro* and *in vivo* populations from all trials. Individual pairwise comparisons (Multiple t-tests) were used to compare the *in vivo* and *in vitro* pathogenicity of each dose. The larval mortality rate was zero in the control group and was not included in the statistical analysis. * indicates low, ** indicates moderate, *** indicates high statistical difference (0.05). ns indicates there was no significant difference. Error bars represent the standard error mean.

Table 1
LD_{50} and LD_{90} values and 95% fiducial confidence intervals of *in vivo* and *in vitro*-produced populations for each parameter.

Parameter	Production	n	Slope ± SE	LD_{50} (95% Fiducial Cl)	LD_{90} (95% Fiducial Cl)	df	Chi^2	*P* value
Temperature (26 °C)	*In vivo*	360	0.07 ± 0.003	13.94 (12.96–14.91)	31.83 (30.06–33.88)	4	81.09	<0.001
	In vitro	360	0.08 ± 0.004	14.19 (13.28–15.11)	30.6 (28.93–32.53)	4	33.12	<0.001
pH (26 °C + pH 9)	*In vivo*	360	0.08 ± 0.003	13.34 (12.40–14.27)	30.29 (28.63–32.21)	4	124.73	<0.001
	In vitro	360	0.11 ± 0.004	11.85 (11.13–12.59)	23.99 (22.71–25.48)	4	14.72	0.005
CO_2 (26 °C + pH 9 + 9% CO2)	*In vivo*	360	0.06 ± 0.002	15.66 (14.59–16.72)	35.62 (33.68–37.85)	4	102.34	<0.001
	In vitro	360	0.11 ± 0.005	9.6 (8.89–10.30)	21.4 (20.16–22.86)	4	14.70	0.005

been made to improve their production processes. In an early improvement study, the highest yield was achieved by modifying the production environment and chemical content (Bedding, 1981). Many subsequent studies have investigated the effect of temperature on yield, and the ideal temperature for *H. indica* was found to be 25 °C, and IJ recovery decreased above 30 °C (Ehlers et al., 2000). In another study, the ideal temperature for the two *Steinernema* species was 25 °C (Hirao and Ehlers, 2009). The yield and morphometric parameters of *S. arenarium* grown at 18 °C and 28 °C were compared, and negative effects were observed at 28 °C (Yakovlev and Kharchenko, 2015). Generally, serious yield losses and deaths were observed at temperatures >25 °C. For instance, the best production temperature for *S. yirgalamense* was 25 °C, and a decrease in yield was observed at 27 °C and 30 °C (Addis et al., 2016a). While the optimal temperature range varies between 18 and 28 °C depending on the EPN species, the highest efficiency was observed at around 25 °C. As in previous studies, the ideal production temperature for the hybrid HBH strain was 26 °C. This is expected to require high temperatures because the parents of the hybrid strain originated in regions with high average temperatures. However, the efficiency decreased at 28 and 30 °C, which is consistent with previous studies, but contrary to the expectations in this study.

The biology of EPNs encompasses a symbiotic relationship involving two distinct organisms: bacteria and nematodes. In the process of *in vitro* production, bacteria are initially cultivated, and subsequently, nematodes develop by feeding on these bacteria. Consequently, the composition of the culture medium is crucial for the symbiotic bacteria. While temperature influences bacterial growth, variations in pH levels can also pose significant challenges. Research has indicated that maintaining a

pH of approximately 7 is optimal for both bacteria and nematodes (Ehlers et al., 2000; Jeffke et al., 2000; Chavarría-Hernández et al., 2006; Addis et al., 2016b). High pH is also known to have a more negative effect than low pH (Shapiro-Ilan et al., 2012). During *in vitro* production, symbiotic bacteria and nematodes alter the pH of the environment owing to their physiological and metabolic activities. Because pH directly affects yield, pH adjustment is also a common practice during production. For instance, pH $\leq$ 4 killed different EPN species, and variable results were obtained at pH 4–9 (Khathwayo et al., 2021; Strauch et al., 2000). Generally, an increase in pH is observed as the production process progresses (Strauch et al., 2000; Gil et al., 2002; Inman et al., 2012). The ideal pH for the hybrid HBH strain was between 7 and 9, which was similar to the results of previous studies. In our study, pH was kept constant at a specific value throughout production to determine the pH effect. In studies without pH adjustment, the pH level rose above 8 towards the end of production. Therefore, we believe that pH adjustment will not be required for future production of hybrid HBH strain.

Since many organisms release CO_2 through respiration, it can have stimulatory effects on other living organisms. Therefore, CO_2 is recognized for its attractive properties. EPNs are likewise known to track traces of CO_2 emitted by the target insect while searching for a host (Ramos-Rodríguez et al., 2007; Banerjee and Hallem, 2020). Moreover, CO_2 in the environment affects the behavior of EPNs (Hallem et al., 2011; Dillman et al., 2012). However, few studies have examined the effects of CO_2 on EPN biology. It is known that, in the presence of food signal in the environment, an increased concentration of CO_2 had a positive effect on IJ recovery (Jessen et al., 2000). In addition, a constant 5% CO_2 concentration in production resulted in an improved yield compared to standard conditions (Ehlers, 2001). Among the four different CO_2 concentrations used in our study, the highest efficiency was obtained at the 8 and 9% concentrations. Considering that high CO_2 concentrations increase the yield, these results are compatible with previous findings (Ehlers, 2001; Jessen et al., 2000). However, the liquid production environment is complex. It is difficult to conclude that the CO_2 concentration alone directly affected the yield. Still, CO_2 affects EPNs either alone or in combination with other factors. Therefore, the effect of CO_2 should be further investigated by developing fermenters and sensitive measurement systems.

A high production efficiency is necessary due to the substantial quantity of EPNs needed for field applications. Simultaneously, it's crucial for the *in vitro* produced nematodes to maintain adequate pathogenicity. The production process has a direct impact on the end product. Consequently, the quality of the product should be assessed after each production cycle. The primary parameter that serves as an indicator of EPN quality following mass production is their pathogenicity. Pathogenicity trials are generally performed using *T. molitor* or *G. mellonella* larvae as in the present study. The pathogenicity of populations produced *in vitro* and *in vivo* has been compared in many studies, and no difference was observed between the production type and control groups (Kong et al., 2022; Neira-Monsalve et al., 2019). These results are consistent with those of the present study. However, *in vitro* and *in vivo* populations do not always exhibit the same pathogenicity. For instance, *in vitro* production can significantly increase the pathogenicity of populations (Dunn et al., 2020), which is consistent with the results of the present study. On the other hand, contrary to these results, the pathogenicity of the *in vivo* populations can be higher than that of the *in vitro* populations (Ferreira et al., 2014, 2016). As a result, the effectiveness of EPNs varies according to species and target insects. Nevertheless, after *in vitro* production, it is necessary to conduct efficacy or pathogenicity trials under field conditions to obtain realistic results (Cortés-Martínez and Chavarría-Hernández, 2020).

The optimization of mass production of EPNs is inevitable to compete with chemical pesticides in terms of both cost and efficacy. Optimization of *in vitro* liquid production is one of the most crucial methods to achieve this (Shapiro-Ilan et al., 2002). The primary objective of this study was to establish an *in vitro* liquid production of the HBH hybrid strain of *H. bacteriophora* by determining the optimal values for temperature, pH, and dissolved CO_2. Identifying the optimal production conditions is a critical step in maximizing efficiency and the potential commercialization of the HBH hybrid strain. Although this study centered on three parameters, future research should incorporate additional variables to broaden the scope and enhance production yields.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants or vertebrates performed by any of the authors.

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

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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