Received: 28 May 2020 | Revised: 15 July 2020 | Accepted: 16 July 2020

DOI: 10.1002/bit.27515

REVIEW

BIOTECHNOLOGY AND BIOENGINEERING WILEY

Production of entomopathogenic nematodes in submerged monoxenic culture: A review

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Funding information
Consejo Nacional de Ciencia y Tecnología, Grant/Award Numbers: CB 2014/238359, INFRA 2014/230138, INFRA 2016/269805, Postdoctoral fellowship 741017/CVU 178235

Abstract

Monoxenic liquid culture is the most suitable technology for scaling up to industrial production of entomopathogenic nematodes (EPNs); however, the variability of the yield production remains a current problem in the process. The aim of this study was to analyze the parameters and criteria for EPN production in liquid culture based on scientific and technological knowledge from the last two decades. While experimental research has permitted the yield production of *Heterorhabditis bacteriophora* (362×10^3 infective juveniles [IJs]/ml) and *Steinernema carpocapsae* (252×10^3 IJs/ml), simultaneously, theoretical approaches have contributed to the understanding of the culture process, based on biological parameters of the bacterium–nematode complex and hydrodynamic and rheological parameters of the complex gas–liquid–solid system. Under this interdisciplinary research approach, bioprocess and biosystem engineering can contribute to design the various control strategies of the process variables, increase the productivity, and reduce the variability that until now distinguishes the in vitro production of EPNs by the liquid culture.

KEYWORDS

biotechnology, *Heterorhabditis*, hydrodynamics, in vitro, liquid culture, *Steinernema*

1 | INTRODUCTION

Entomopathogenic nematodes (EPNs) of the *Heterorhabditidae* and *Steinernematidae* families are obligate and lethal endoparasites of insects due to their symbiotic association with *Photorhabdus* and *Xenorhabdus* bacteria, respectively (Lewis & Clarke, 2012). The so-called infective juvenile (IJ) is the only free-living stage of EPN and typically occupies soil habitats until it infects an insect, allowing to resume its life cycle and produce new progeny (Behle & Birthisel, 2014; Grewal & Peters, 2005). The EPNs are natural regulators of soil insect populations (Ferreira & Malan, 2014) which do not represent a risk for human and animal health but are highly specific (Boemare, Laumond, & Mauleon, 1996). By these advantages, EPNs are of interest for their application as biological control agents of insect pests in agriculture.

The successful commercialization of EPNs as a biopesticide product versus a chemical insecticide product depends on industrial mass production of viable IJs at low cost (Ehlers, 2001), maintenance of infectivity, and extension of shelf life through the formulation (Cortés-Martínez, Lewis, Ruiz-Vega, & Martínez-Gutiérrez, 2017; Cruz-Martínez, Ruiz-Vega, Matadamas-Ortíz, Cortés-Martínez, & Rosas-Diaz, 2017; Peters, 2016). Various authors argue that the submerged monoxenic culture in bioreactors is the most suitable technology for scaling up to profitable industrial production of EPNs, because the yield process is high, although variable (Abu Hatab & Gaugler, 1999; Belur, Inman, & Holmes, 2013; Shapiro-Ilan & Gaugler, 2002; Shapiro-Ilan, Han, & Dolinski, 2012).

According to the criteria for EPN application in integrated pest management programs, the minimum recommended rate for inundative applications is 2.5×10^9 IJs/ha (Shapiro-Ilan et al., 2012). Therefore, under current yield rates (of the order of 460×10^3 IJs/ml), industrial production in 5.4 L batches or higher is required to provide the necessary nematode supplies for 1 ha of cultivation area. For achieving this goal, the greatest challenge in submerged monoxenic culture is to maintain the growth of the bacterial–nematode complexes according to its changing requirements and technological restrictions, providing

optimal conditions for reproduction differentially, which remains an engineering problem in the on-going solution process.

The biotechnological challenge to improve the performance of the culture process makes it necessary the control of physiology of symbiotic bacteria, formulation of culture medium (Chavarría-Hernández, Espino-García, Sanjuan-Galindo, & Rodríguez-Hernández, 2006; Islas-López, Sanjuan-Galindo, Rodríguez-Hernández, & Chavarría-Hernández, 2005; Leite, Shapiro-Ilan, Hazir, & Jackson, 2016a; Masurekar, 2008; Yoo, Brown, Cohen, & Gaugler, 2001), and process parameters, such as oxygen transfer rate, temperature, pH (Ferreira, Addison, & Malan, 2016), inoculum size and timing of inoculation (Johnigk, Ecke, Poehling, & Ehlers, 2004), EPN sensitivity to shear and normal stresses, IJ recovery, geometry, and configuration of the bioreactor (Chavarría-Hernández, Sanjuan-Galindo, Rodríguez-Pastrana, Medina-Torres, & Rodríguez-Hernández, 2007; Neves, Simões, & Mota, 2001) among others.

From 1980 to 2010, 65 articles on culture medium and parameters for mass production of EPNs were published (San-Blas, 2013). Since the study by Buecher and Popiel (1989) for in vitro production of *S. feltiae*, the development of the technique for liquid culture has been one of the most significant advances. Experimental results and theoretical analyses of the propagation kinetics of *S. feltiae*, the effect caused on nematodes by hydrodynamics and oxygen transfer is discussed for first time in the review of Lopez-y-Lopez, Chavarría-Hernández, Fernández-Sumano, and De la Torre (2000) and De la Torre (2003). Ehlers (2001) did a state-of-knowledge review for analyses of technology and techniques for the production of nematodes in submerged culture in bioreactors, and 11 years later, Inman, Singh, and Holmes (2012) published an analysis review based on the available information for the production of *Heterorhabditis bacteriophora* Poinar. The review chapter of Peters, Han, Yan, Leite, and Lacey (2017) discusses the development of the *P. luminescens–H. bacteriophora* complex in liquid culture caused by variations in temperature, pH, stirrer speed, and the percentage of dissolved oxygen (DO) saturation, physical parameters collected in production processes at industrial scale from the company e-nema GmbH.

If submerged culture is the technological solution for mass production of nematodes, it is partly since the biology of various nematode–bacterial complexes, and the process parameters for reproduction and growth have been studied empirically and theoretically, and the technique of submerged cultivation has been systematized and simplified. Therefore, the objective of this study is to analyze the theoretical and empirical studies published in the last two decades and to discuss the progress on the criteria, variables, and parameters of the liquid culture process for the growth and reproduction of bacteria–nematode complexes.

2 | DATABASE

First, a search with the keywords *Steinernema* or *Heterorhabditis*, and *Xenorhabdus* or *Photorhabdus*, and "liquid culture" or "production" was done in the Web of Science™ Core Collection from the period 2000–2020. The selected publications were those whose abstract made explicit that the production of EPNs was by the submerged culture. A metadatabase of 46 published articles in Bibtex format was processed with the Bibliometrix software package (Aria & Cuccurullo, 2017) and built-in R-studio to conduct the scientific mapping analysis. Figure 1 shows the conceptual structure of a framework using a keyword co-occurrence network that expresses common concepts that we highlight as general topics of research for EPN production by the submerged monoxenic culture and four thematic clusters were identified.

The map in Figure 1 represents the research field, and the keyword "mass production" is near the center of the research field as a large number of the published articles focused on this study area. The four thematic clusters were yield production (red), selective breeding (blue), reproductive biology (green), and applications on insect pest (purple). The first cluster groups most of the papers and refers to process parameters, some of which have been experimental and theoretical studies focused on the operation of the bioprocess. The second cluster refers to experimental strategies to maintain the EPN fitness or to decrease their deterioration. The third cluster is related to studies on fundamental reproductive biology and population dynamics of EPNs through the in vitro liquid culture technique. Finally, the fourth cluster groups studies on control effectiveness on insect pest under field and laboratory conditions. Table 1 shows the articles corresponding to each cluster.

Table 2 presents the top 5 rankings of authors (columns 2 and 3) and countries (columns 4 and 5) concerning the total number of citations (TC) and publications (NP). Ehlers from Germany stands as the leading author in terms of TC and NP. In America, Chavarría-Hernández stands as lead author in NP (10) and Mexico as the leading country in TC and NP. Rodríguez-Hernández is a top researcher focused on rheological characterizations of liquid broths for EPN production. The top 5 rankings of liquid culture-EPN publications in terms of TC and average citations per year (ACY) in descending order are Susurluk and Ehlers (2008) (TC: 47, ACY: 3.62), Han and Ehlers (2001) (TC: 38, ACY: 1.90), Yoo et al. (2000) (TC: 31, ACY: 1.48), Kurtz et al. (2009) (TC: 30, ACY: 2.5), and Johnigk et al. (2004) (TC: 30, ACY: 1.76).

In the following sections, the collected documents were analyzed according to the general research topics: nematode–bacteria relationship and production process of the nematode–bacterial complex. During the analysis, other studies from Scopus®, US patents, South Korea patents, and WIPO patents databases were included (research articles, review articles, book chapters, and patents) that were considered relevant for the discussion of the established general topics, regardless of their publication date.

3 | BACTERIA–NEMATODE RELATIONSHIP

3.1 | Bacteria–nematode life cycle

The life cycle of *Heterorhabditis* spp. with alternative development pathways during the in vitro liquid culture is reported by

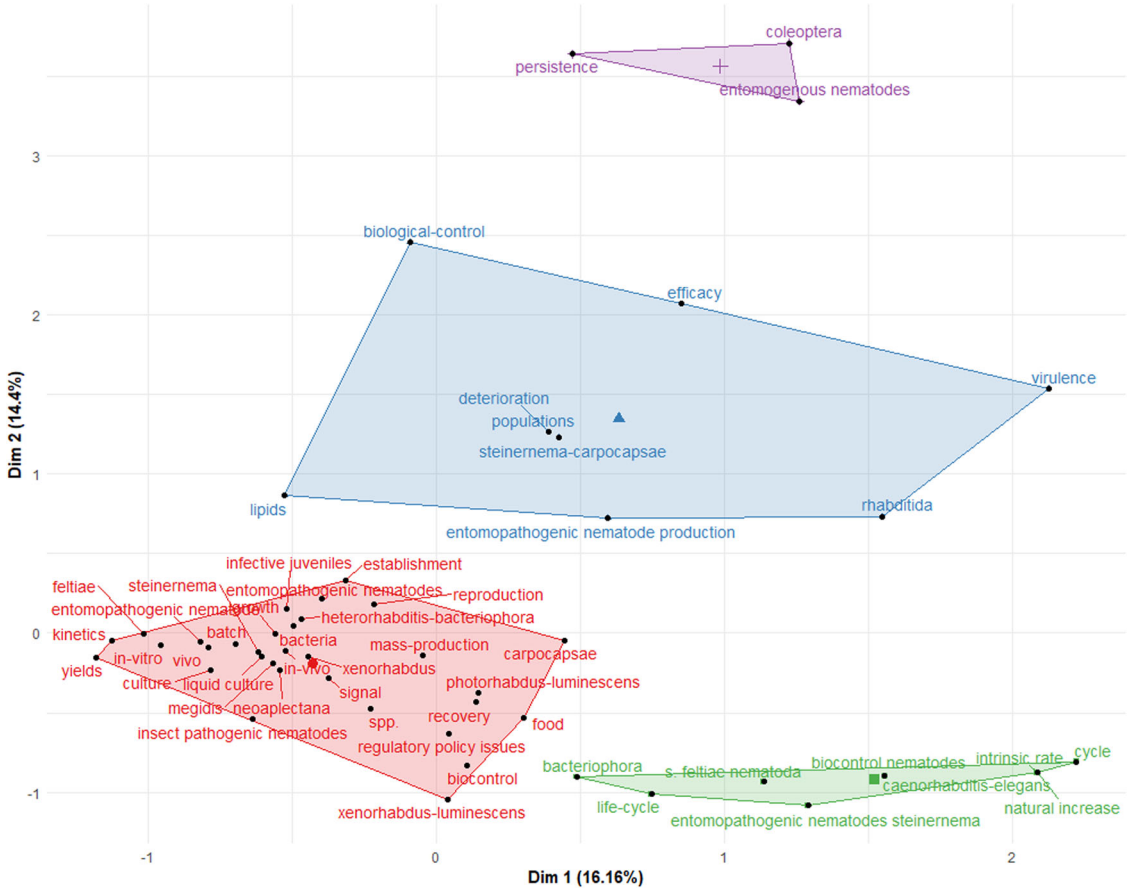

FIGURE 1 Conceptual map and clusters of keywords in 46 documents from Web of Science™ Core Collection related to liquid monoxenic culture of entomopathogenic nematodes (EPNs) from the period 2000–2020 [Color figure can be viewed at wileyonlinelibrary.com]

Johnigk and Ehlers (1999), Strauch and Ehlers (2000), and Ehlers (2001), but there are no reports of the life cycle of *Steinernema* spp., while is produced by this technique. The life cycle of *Xenorhabdus* or *Photorhabdus* is unique because they are symbiotic with nematodes and pathogenic with insects (Boemare, 2002). Figure 2 shows a graphical representation of the general life cycle of the bacteria–nematode complex, according to Forst and Clarke (2002), Shi and Bode (2018), and Herbert and Goodrich-Blair (2007).

As can be seen in Figure 2, the cyclical association of the symbiosis of bacterium–nematode complexes is explained as a three-stage process (Forst & Clarke, 2002). In Stage I, the bacteria cells are retained in the IJ intestine while it remains in the soil and until it enters the insect hemocoel. In early Stage II, bacteria are released by the nematodes in the insect hemolymph (by defecation in the case of *Xenorhabdus* or by regurgitation in the case of *Photorhabdus*) to produce virulence factors that cause a fatal infection. At the same time, IJ recovery occurs to restart its development. In late Stage II, the growth phase of the bacterium occurs at a high cell density and the bioconversion of the insect cadaver to support the individual development and nematode growth. In Stage III, the nematodes become an alternative J3 stage known as the larva dauer when food is scarce, and the density of nematodes is high. Then, bacterial colonization of IJs begins, *Xenorhabdus* present in high density colonizes a specialized bilobed intestinal vesicle and is retained with the help of the flagella present in the cells of the Phase-I bacteria; meanwhile, *Photorhabdus* colonizes the anterior region of the intestine posterior to the basal bulb (Ferreira & Malan, 2014).

The complex shows three modes of symbiosis: (a) offensive mutualism to create the conditions for the bacteria in the early infection stage, (b) resource harvest mutualism of nutritional collection, and (c) defensive mutualism—the later one to protect the host cadaver during late growth stages. Notably, in the symbiotic mutualism of resource collection, it is argued that if Phase II proliferates in the host, the metalloprotease enzyme that facilitates the bioconversion of the host tissue is produced scarcely, resulting in a low-yield production of nematodes (Koppenhöfer & Gaugler, 2009), which agrees with the observed low IJ yield in liquid cultures with secondary phase bacteria. The bacteria–nematode association is discussed in more detail by Koppenhöfer (2007), and Ferreira and Malan (2014) review the biology of the complex in terms of the optimal growth parameters in liquid culture.

TABLE 1 Published articles into each thematic cluster identified in the research field of submerged culture of entomopathogenic nematodes during the period 2000–2020, from the Web of Science™ Core Collection

Red cluster	Blue cluster	Green cluster	Purple cluster
Neves, Teixeira, Simoes, and Mota (2001)	Anbesse et al. (2012)	Addis et al. (2014); Addis, Teshome et al. (2016)	Kurtz, Hiltpold, Turlings, Kuhlmann, and Toepfer (2009)
	Anbesse, Sumaya, Dörfler, Strauch, and Ehlers (2013a, 2013b)	Addis, Demissie et al. (2016)	
		Addis, Mijušković et al. (2016)	
Kim, Kim, Yasunaga-Aoki, and Yu (2014)	Sumaya et al. (2018)	Ehlers et al. (2000)	Shapiro and McCoy (2000)
Chavarría-Hernández and De la Torre (2001)	–	Ferreira, Addison, and Malan (2014)	Susurluk and Ehlers (2008)
Chavarría-Hernández et al. (2003, 2006, 2007, 2010, 2011, 2014); Chavarría-Hernández, Islas-López, Maciel-Vergara, Gayosso-Canales et al. (2008); Chavarría-Hernández, Islas-López, Maciel-Vergara, Pastrana et al. (2008)		Ferreira et al. (2016)	
Cho, Whang, Gaugler, and Yoo (2011)	–	–	Steyn, Malan, and Addison (2019)
Gil, Choo, and Gaugler (2002)	–	–	–
Han and Ehlers (2001)	–	–	–
Hirao and Ehlers (2009a, 2009b, 2010)	–	–	–
Hirao, Ehlers, and Strauch (2010)	–	–	–
Islas-López et al. (2005)	–	–	–
Jessen, Luttmann Ehlers, Strauch, and Wyss (2000)	–	–	–
Johnigk, Hollmer, Strauch, Wyss, and Ehlers (2002) Johnigk et al. (2004)	–	–	–
Leite et al. (2016a) Leite, Shapiro-Ilan, Hazir, and Jackson (2016b, 2017)	–	–	–
Pérez-Campos, Rodríguez-Hernández, López-Cuellar, Zepeda-Bastida, and Chavarría-Hernández (2018) Pérez-Campos et al. (2019)	–	–	–
Strauch and Ehlers (2000)	–	–	–
Young et al. (2002)	–	–	–
Yoo, Brown, and Gaugler (2000) Yoo et al. (2001)	–	–	–

TABLE 2 Top 5 rankings of liquid culture-EPN authors and countries in terms of total citations and number of publications

	Authors		Countries	
Rank	TC	NP	TC	NP
1	Ehlers R. U. (318)	Ehlers R. U. (17)	Germany (280)	Germany (16)
2	Strauch O. (163)	Strauch O. (11)	Mexico (87)	Mexico (11)
3	Gaugler R. (87)	Chavarría-Hernández N. (10)	USA (86)	USA (5)
4	Chavarría-Hernández N. (84)	Rodríguez-Hernández A. I. (9)	China (38)	South Africa (3)
5	Hirao A. (58)	Gaugler R. (5)	Hungary (30)	Brazil (3)

Abbreviation: EPN, entomopathogenic nematode.

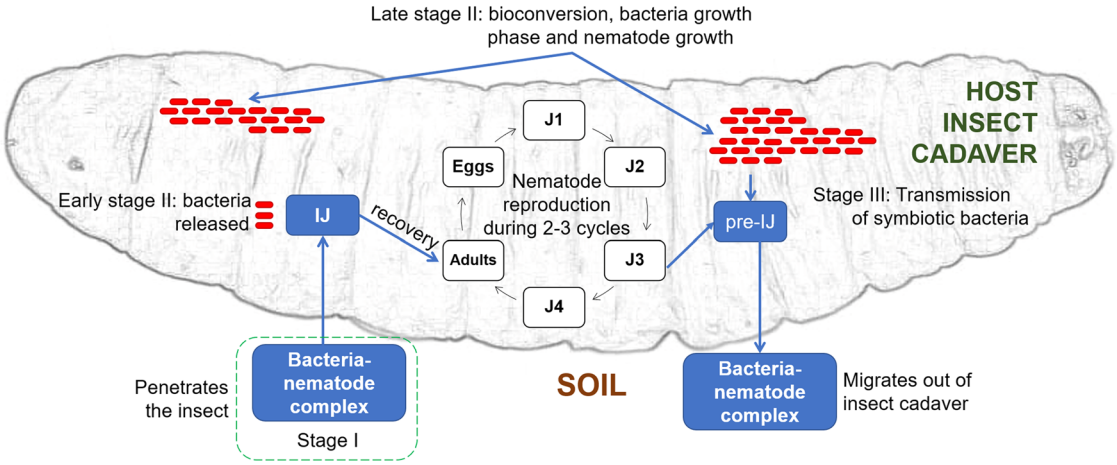

FIGURE 2 The life cycle of bacterium–nematode complexes during infection and reproduction on host insect cadaver [Color figure can be viewed at wileyonlinelibrary.com]

3.2 | Isolation of symbiotic bacteria

The first action to establish the liquid culture is the isolation of the symbiotic bacteria, *Photorhabdus* spp. or *Xenorhabdus* spp., in the primary phase because it has a superior ability to support the nematode propagation in monoxenic liquid culture, in most of the cases (Han & Ehlers, 2001) and effective killing of the target insect pests. These bacteria can be obtained from (a) hemolymph extracted of superficially sterilized last instar of wax moth *Galleria mellonella* previously infected by IJs, (b) crushed surface-sterilized IJs, or (c) an insect hemolymph drop cultured with IJs. Afterward, a sample is spread onto a selective/differential medium for isolation of primary phase. Koppenhöfer (2007) reports these methods in more detail for the isolation and characterization of symbiotic bacteria. It is crucial to consider that the characteristics of Phase-I bacterial colonies (color, diameter, or growth rate) vary in each medium for isolation (Table 3). They can even present different color shades among strains of the same species.

The axenic growth of the bacterium, *Xenorhabdus* spp. or *Photorhabdus* spp. lasts approximately 48 hr. At the beginning of its development, a lag phase of 0–15 hr occurs. The exponential phase begins after 15 hr, followed by the stationary phase after 42 hr (Ferreira et al., 2016; Yoo et al., 2001). However, this growth timing is not a general rule. Orozco-Hidalgo, Quevedo-Hidalgo, and Sáenz-Aponte (2019) reported three metabolic phases of *P. luminescens* subsp. *akhurstii* SL0708. The primary phase lasted 36 hr, the intermediate phase occurred from 36 to 72 hr, and the secondary phase was observed from 72 to 96 hr.

TABLE 3 Characteristic pigmentation of some Phase-I symbiotic bacteria streaked on two differential selective media, NBTA or MacConkey agar

Host nematode	Symbiotic bacteria	NBTA	MacConkey agar	Reference
S. carpocapsae	*X. nematophilus*	Blue	Brown	Akhurst and Boemare (1988)
S. glaseri, *S. cubanum*	*X. poinarii*	Red	Red	Akhurst and Boemare (1988)
S. colombiense	*X. nematophilus*	Blue	NR	Pérez-Campos et al. (2018)
S. feltiae, *S. intermedium*, *S. kraussei*, *S. affine*	*X. bovienii*	Blue	Brown	Akhurst and Boemare (1988)
S. riobravis	*X. cabanillasii*	Blue	Red	Cabanillas et al. (1994)
S. scapterisci	*X. innexi*	Red	NR	Lengyel et al. (2005)
S. yirgalemense	*X. indica*	Blue	NR	Ferreira, van Reenen et al. (2014)
H. bacteriophora	*P. luminescens*	Green	Red or brown	Akhurst and Boemare (1988)
H. indica	*P. luminescens*	Green	Red	Thomas and Poinar (1979) Boemare, Akhurst, and Mourant (1993)

Abbreviation: NR, not reported.

3.3 | Quality of in vitro produced entomopathogenic nematodes

The EPN quality is usually defined in terms of their viability, virulence, and morphometry. For some time, it was believed that the quality of IJs in vitro produced was lower than that in-vivo reared (Shapiro-Ilan et al., 2012), particularly if the natural host insects of each species of nematodes are used. However, the distinction of viability and virulence of IJs conventionally produced by both methods is marginal under laboratory conditions (Converse & Miller, 1999; Grewal, Converse, & Georgis, 1999).

EPNs produced in liquid culture are equally effective, for example, in applications of *S. carpocapsae* (Weiser) on *Popillia japonica* Newman and *Otiorhynchus sulcatus* (F.) (Gaugler & Georgis, 1991), *S. jeffreyense* and *S. yirgalemense* on *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae; Steyn et al., 2019), of *S. riobrave* against *Diaprepes abbreviatus* (Shapiro & McCoy, 2000), of *H. bacteriophora*, *H. megidis* or *S. feltiae* against *Diabrotica virgifera* (Kurtz et al., 2009) or *S. feltiae* against *Lucilia cuprina* (Pace, Grote, Pitt, & Pitt, 1986). Even the prolonged persistence of *H. bacteriophora* in vitro produced was detected 23 months after their application in bean crops, followed by rotation by wheat, with red clover as a cover crop (Susurluk & Ehlers, 2008).

Therefore, the premise that the efficacy of control depends on the selection of an appropriate EPN species or strain for the target insect pest is valid if nematodes are in vitro produced. Although the bioassay on the infection model insect *G. mellonella* is a well-accepted technique to determine the virulence profiles of an IJ population (Converse & Miller, 1999), it is necessary to complement the in vitro production studies with effectiveness evaluations of nematodes on target pest insects, under controlled, semi-controlled, and field conditions.

4 | THE PRODUCTION PROCESS OF BACTERIA–NEMATODE COMPLEX

4.1 | Liquid culture

The process of monoxenic liquid culture of EPNs comprises two stages. In the first stage, a Phase-I bacterial culture broth in its exponential growth stage is inoculated into a sterile medium at a concentration of 0.5–5% (v/v) for *Photorhabdus* and 1–11% (v/v) for *Xenorhabdus* at 10^7–10^{10} cells/ml and is incubated for 24–48 hr (based on analysis of 46 selected publications). During this step, the symbiotic bacteria support (a) the conversion of the medium into accessible components for both bacteria and nematodes (Chavarría-Hernández et al., 2006), (b) the secretion of metabolites (Inman et al., 2012), and (c) serve as the primary food source for development and reproduction of nematode (Gaugler & Han, 2002).

Regarding the liquid culture of the bacteria–nematode complex, the growth kinetics of the bacteria *Xenorhabdus* spp. or *Photorhabdus* spp. have been investigated. The specific growth rate (μ) of *P. luminescens* during the exponential phase has been modeled through time (t) by Equation (1) (Castillo, 1995)

$$\mu = (\ln x_1 - \ln x_0)/(t_1 - t_0), \tag{1}$$

where x_1 and x_0 are the bacterial biomass concentrations (g/L) at times t_1 and t_0, respectively. In the second stage of the process, sterile IJs are inoculated for cocultivation of nematodes and bacteria (Gil et al., 2002; Yoo et al., 2001). The first mathematical approach to model the population growth (C) of steinernematids in liquid culture was conducted by Chavarría-Hernández and De la Torre (2001), using a reparameterized Gompertz model (Zwietering, Jongenburger, Rombouts, & Van't Riet, 1990).

$$\frac{C}{C_0} = \left(\frac{C}{C_0}\right)_{\max} \times \exp\left\{-\exp\left[\frac{m_{max} \times e}{(C/C_0)_{\max}}(\lambda - t) + 1\right]\right\}. \tag{2}$$

The model involves the following parameters: (a) time required for the IJ to reach the adult stage (λ), (b) EPN multiplication factor, $(C/C_0)_{max}$, where C_0 is the initial total IJ concentration, and (c) maximum population growth rate ($m_{max} = \{d(C/C_0)/dt\}_{max}$). This model has been used for the data analysis in in vitro production studies of *S. feltiae* (Mexican; Chavarría-Hernández & De la Torre, 2001), *S. carpocapsae* (Mexican; Chavarría-Hernández et al., 2006; Chavarría-Hernández, Islas-López, Maciel-Vergara, Gayosso-Canales, & Rodríguez-Hernández, 2008; Chavarría-Hernández, Islas-López, Maciel-Vergara, Pastrana, & Rodríguez-Hernández, 2008), and *S. colombiense* (Pérez-Campos et al., 2018), with good results.

One of the operating premises of the process is that an optimum culture temperature would be reached to promote bacterial growth to avoid a phase change, which generally causes a low-yield. Ferreira and Malan (2014) suggest that the factors that determine the optimal temperature are both bacteria–nematode complex and culture medium composition. In the culture of *P. luminescens–H. indica* complex, the IJ recovery was higher at 25°C (24%); therefore, a low number of hermaphrodites were developed at 27°C (15%) and 30°C (6%). However, after 14 days, there were no significant differences in the mean IJ yield, and it was speculated that the low density of hermaphrodites was compensated by an increase in the offspring production by hermaphrodite (Ehlers et al., 2000). Then, IJ recovery is not a definitive indicator of final IJ concentration. In the case of steinernematids, the temperature of culture is not related to the IJ recovery, but it has been observed that at low temperatures, the EPN development is delayed. Based on the performance of IJ and its proportion in the final population of nematodes, it was observed that the optimal temperature for the culture of *S. carpocapsae* and *S. feltiae* was 25°C (Hirao & Ehlers, 2009a).

4.2 | Infective juvenile yield

In the last two decades, eight steinernematids and five heterorhabditids have been evaluated in the monoxenic liquid culture, at

laboratory or pilot scales. The yield of heterorhabditids was from 280×10^3 to 457×10^3 IJs/ml in Erlenmeyer flask cultures of 3 weeks (Ehlers et al., 2000; Shapiro-Ilan & Gaugler, 2002). In a 7-L bioreactor, the reported yield for *H. bacteriophora* was $39–46 \times 10^3$ IJs/ml (Upadhyay et al., 2015). On a pilot scale, the first studies reported yields of the order of 100×10^3 IJs/ml in 20-L fermenters pneumatically agitated in processes of 15–20 days, while in a 500-L fermenter, the maximum yield was 105×10^3 IJs/ml in a 60-day process (Surrey & Davies, 1996). The liquid culture on an industrial scale of 8,000 L involved 10 days of fermentation at the company e-nema GmbH (Peters et al., 2017).

A laboratory or industrial scale, the production of steinernematids in liquid media is also variable. In a 4.22-L bioreactor with pneumatic agitation and variable aeration rate, *Q*, for the mass culture of *S. carpocapsae* CABA01, the IJ yield and the duration time were 252×10^3 IJs/ml and 16 days, respectively (Chavarría-Hernández et al., 2011). Table 4 summarizes the nematodes produced in monoxenic liquid culture, the involved productivity, and the technology used.

Mathematical modeling and computational simulation as tools to predict the process yield have been explored very less in liquid culture of EPNs. Addis, Teshome, Strauch, and Ehlers (2014) and Addis, Teshome, Strauch, and Ehlers (2016) studied the life history of *S. riobravis* and *S. feltiae* following the hanging drop method. Life-cycle parameters were calculated by iteration of the intrinsic ratio of natural increase (r_m) by the Euler/Lotka equation.

$$\sum_{x=0}^{d} e^{-r_m x} l_x m_x = 1, \tag{3}$$

where x is the time in days, l_x is the specific survival probability by age, and m_x is the specific fecundity by age. By the use of this equation, the population growth of *S. riobravis* and *S. feltiae*, and the reproductive, and development parameters were well modeled. Their results show that offspring production increases if bacterial food density increases. The later maintains a positive correlation with (a) the total number of offspring that would be produced by the female if they were able to survive until the end of their reproductive period, and (b) the average number of offspring produced by a female during its lifetime, dependent on the age-specific survival probability, and fecundity of the female.

An important observation by Addis, Teshome et al. (2016) is that the mean body volume of the females and the number of parental females 3 days after inoculation would allow predicting the final yield of *S. feltiae* IJs. However, the validity of this modeling has yet to be tested in the production of other EPNs. For example, in the liquid culture of *H. bacteriophora* in 250-ml flasks incubated at 25°C and 200 rpm, where it was found that the size of the first-generation hermaphrodites was correlated with the yield of the process (Yoo et al., 2001).

4.3 | Equipment and innovations

At laboratory scale, liquid culture is carried out using different flasks, tissue culture bottles, and mechanically or pneumatically agitated bioreactors. The production in a 20-L stirred tank reactor has been reported by Friedman, Langston, and Pollitt (1989), Pace et al. (1986),

TABLE 4 Data of productivity of IJs obtained in submerged monoxenic culture of entomopathogenic nematodes, *Steinernema* and *Heterorhabditis*, in reports published from 1986 to 2020

Entomopathogenic nematode	Final IJ concentration ($\times 10^3$ IJs/ml)	IJ productivity[a] ($IJs \cdot ml^{-1} \cdot day^{-1}$)	Technology used	Agitation method	Reference
H. bacteriophora	362	29,667	Flask	Orbital shaker	Gil et al. (2002)
H. indica	457	26,353	Flask	Rotary shaker	Ehlers et al. (2000)
H. zealandica	41.1	2,607	Flask	Orbital shaker	Ferreira, Addison et al. (2014)
H. megidis	71.47	44,000	Bioreactor	Paddle impeller	Kim et al. (2014)
H. heliothidis	20	DMC	Flask	Orbital shaker	Pace et al. (1986)
S. carpocapsae	252	15,714	Bioreactor	Pneumatic	Chavarría-Hernández et al. (2011)
S. feltiae	225	7,857	Flask	Orbital shaker	Leite et al. (2017)
S. riobravis	NR	DMC	NR	NR	Shapiro and McCoy (2000)
S. colombiense	53	4,990	Flask	Orbital shaker	Pérez-Campos et al. (2018)
S. scapterisci	NR	DMC	NR	NR	Grewal et al. (1999)
S. jeffreyense	121	8,560	Flask	Orbital shaker	Dunn, Belur, and Malan (2020)
S. yirgalemense	75	4,733	Flask	Orbital shaker	Ferreira et al. (2016)
S. glaseri	200	DMC	Flask	Orbital shaker	Park and Song (2003)
S. bibionis	70	DMC	Flask	Orbital shaker	Pace et al. (1986)

Abbreviations: DMC, data missing to calculate; IJ, infective juvenile; NR, not reported.
[a]Calculated with the formula ($C_{IJ,\ final} - C_{IJ,\ 0}$/liquid culture span).

and Surrey and Davies (1996). The mixing is provided with axial impellers as Kaplan turbines (Ehlers, Lunau, Krasomil-Osterfeld, & Osterfeld, 1998), marine propeller (Strauch & Ehlers, 2000), custom-design impellers (Chavarría-Hernández, Ortega-Morales, Vargas-Torres, Chavarría-Hernández, & Rodríguez-Hernández, 2010), paddle impeller (Kim et al., 2014), or radial agitators as Rushton turbine (Belur et al., 2013; Figure 3).

However, the shear sensitivity of EPNs is the limited step to achieve an adequate aeration by mechanical agitation. Therefore, maintaining a homogeneous distribution of nematodes in vessels, and providing enough oxygen supply at acceptable shear forces has motivated the use of geometric modifications to cause vertical circulation of culture broth. In mechanically agitated airlift bioreactors, the mixing is favored by the use of proper impellers and an inner cylinder (Chavarría-Hernández et al., 2007, 2010, 2011; Chisti & Jauregui-Haza, 2002; Ehlers et al.,1998; Strauch & Ehlers, 2000). In pneumatically agitated airlift bioreactors, the flow-through internal or external channels is due to the gas injection (Neves, Simões et al., 2001; Neves, Teixeira et al., 2001).

Attending the usability, on-site use, and low-cost requirements, a disposable system consisted of an airlift microfermenter for mass production of EPNs provided with a kit for culturing to be used by inexpert individuals was patented by Gaugler and Abu Hatab (2002). But the IJ productivity of this technology compared with pneumatic or mechanically agitated tanks is uncertain, thus, is not possible to determine the real benefit in EPN production technique. Nevertheless, although mechanical agitation technology is coarse (Hemrajani & Tatterson, 2004), experimental research in large-, medium-, or small-scale EPN production using innovative mixing approaches is still marginal.

4.4 | Media and additives

One design goal for liquid culture processes is to formulate culture media whose composition provides the adequate nutrients for bacteria and nematodes in optimal concentrations to avoid the reduction of its physiological quality, maintaining its effectiveness (Abu Hatab & Gaugler, 1999; Womersley, 1993; Yoo et al., 2000, 2001; Zhen et al., 2018) and optimize the yields (Ehlers, 2001; Leite et al., 2016a). The culture medium for bacteria and EPNs is a complex mixture that must include convenient amounts of macroelements (i.e., carbon, oxygen, hydrogen, nitrogen, sulfur, and phosphorus), micronutrients (i.e., manganese, zinc, cobalt, molybdenum, nickel, and cooper), and growth factors (i.e., amino acids, purines and pyrimidines, and vitamins), to support the growth of these chemo-organoheterotrophic organisms (Prescott, Harley, & Klein, 2002). Media are usually adjusted around pH 7. Silicon emulsion is added in bioreactor cultures to avoid the generation of foam during the aeration at maximum 0.20% (v/v), depending on the condition of the media. To avoid contamination, cultures can receive ampicillin, streptomycin, or other antibiotics. Table 5 shows the ingredients usually reported to formulate culture media.

It has been demonstrated that a high concentration of ingredients could create a high osmotic pressure, which would significantly reduce the growth of bacteria and nematodes (Leite et al., 2016b). In the liquid culture of *H. bacteriophora–P. luminescens* complex, Yoo et al. (2001) report that the concentration of components in the culture medium and the IJ recovery percentage follow an increasing linear relationship up to an optimal value (84 g/L), after which the relationship was inverse linear. The same relationship trends show the concentration versus bacterial biomass and the concentration versus the size of first-generation hermaphrodites.

Research on alternative ingredients for EPN production has led to the evaluation of agroindustrial by-products as a cheap source of nutrients for nematode–bacteria complex. Important multiplication factor (632) and maximum concentration of viable juveniles (249×10^3 IJs/ml) were achieved using 8–28% (v/v) agave juice (aguamiel) from Mexican maguey-pulquero (*Agave* spp.), as a primary carbohydrate source for *S. carpocapse* (Islas-López et al., 2005). Using whey (a by-product from the dairy industry) for the culture medium formulation, the multiplication factor and maximum concentration of viable juveniles were 298 and 126×10^3 IJs/ml, respectively. The authors suggest that the carbohydrates were mainly used by *X. nematophila* (because of having the capacity for lactose hydrolysis), while the nitrogen source was mainly consumed by *S. carpocapsae* (Chavarría-Hernández et al., 2006). Sterilized supplements from

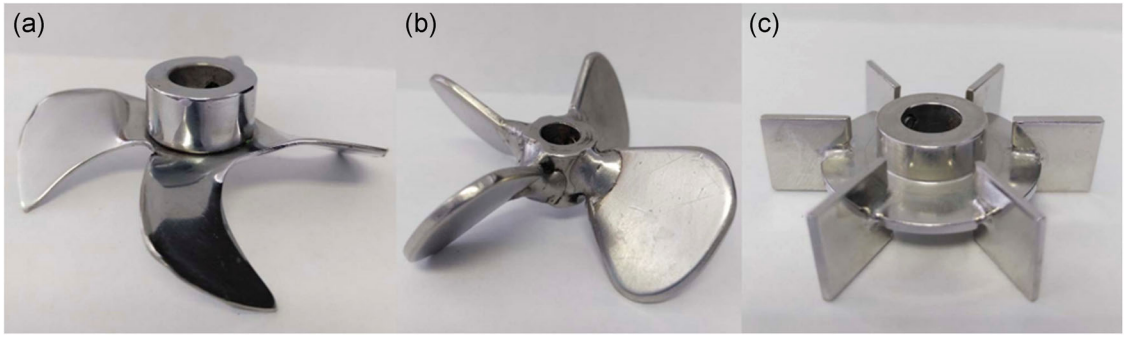

FIGURE 3 Examples of impellers used in liquid culture of nematode–bacteria complexes in bioreactors. (a) Inclined paddle impeller with sharp end, (b) Inclined paddle impeller with rounded edges, and (c) Rushton turbine [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 5 Ingredients used to formulate culture media for submerged monoxenic culture of EPNs

Origin	Ingredients	Function and/or source of
Animal	Dried egg yolk	Cholesterol and proteins, emulsifier
	Pork lard	Lipid
	Whole milk powder	Protein
	Nutrient broth	Protein
	Liver extract	Vitamins B and G
	Beef peptone	Protein and nitrogen
	Hemoglobin	Iron
	Lactalbumin hydrolysate	Haem cofactor and physiological iron
	Lecithin	Emulsifier
	Casein	Protein
Vegetal	Oil (corn, rapeseed, sunflower, thistle, canola, olive, palm, salmon, peanut, sesame, coconut, or soybean)	Lipid
	Soy flour	Protein
	Trypticase soy broth	Amino acids and energy
	Sap from *Agave* spp.	Carbohydrate, minerals, and growth factors
	Lecithin	Emulsifier
Mineral	KCl	Enzymatic
	$CaCl_2$	Calcium supplement
	NaCl	Binder and maintains the osmotic equilibrium
	$MgSO_4$	Magnesium
	$FeSO_4$	Iron
Microbial	Yeast extract	Nitrogen, peptides, and mineral salts
By-products	Whey from the dairy industry	Carbohydrate, proteins, and minerals

Abbreviation: EPN, entomopathogenic nematode.

insect cadavers (i.e., *G. mellonella*), infected or not infected with EPNs, incorporated in the culture of *S. feltiae* or *S. carpocapsae*, have rendered good yields (Cao et al., 2013; Fuchi, Ono, Kondo, & Yoshiga et al., 2016; Zhen et al., 2018), suggesting that the cadavers provide heat-stable nutrients and are therefore of scientific interest to explore its benefits on IJ yield of monoxenic liquid culture.

It is known that the nutritional quality of the liquid culture medium decreases when the density of EPN increases. In this sense, low food availability is an accepted cause for the formation of IJ stage, but some authors suggest that a harmful growth environment could be a second cause and that the emergency is due to repulsion to accumulated ammonia that comes from feces of the EPN into the host cadaver (San-Blas, Gowen, & Pembroke, 2008; San-Blas, Pirela, García, & Portillo, 2014). Therefore, a current question is about the effect of ammonium and other compounds present in the culture broth on the viability of the EPN and the yield process. It is pertinent to develop the control technique for this and other compounds due to its effects in the whole process.

Leite et al. (2016b) suggest that the combination of a viscosifier agent with a low orbital stirring speed (approx. 180 rpm) improves the homogeneous distribution of oil and air in liquid medium contained in Erlenmeyer flask, makes lipids available and improves oxygen transfer to microorganisms. But, the benefit of incorporating a viscosifier agent could be more due to the hydrodynamics and oxygenation promoted than the distribution of nutrients in the medium, because at moderated (>10 mPa·s) and at elevated (>80 mPa·s) media viscosities a thick liquid film of low slipping speed is formed on shake flask wall and the maximum oxygen transfer rate (OTR_{max}) improves, as Giese et al. (2013) suggest.

4.5 | Rheological behavior of the culture medium

The EPN culture broth is a gas–liquid–solid system. In the first approach, nematodes were considered the solid phase, the dissolved nutrients in the broth, the liquid phase, and the air bubbles the gaseous phase (Neves, Simões et al., 2001). However, recent studies

show that the liquid phase contains ingredients suspended at the beginning of the process, such as 350-μm coagulate egg yolk particles and vegetal oil droplets (Pérez-Campos et al., 2019). Also, this is a heterogeneous system due to a changing size of nutrient particles through the processing time, hence, its rheological behavior changes in time.

Chavarría-Hernández, Rodríguez-Hernández, Perez-Guevara, and De la Torre (2003) formulate a liquid medium composed of yeast extract, dehydrated egg yolk, NaCl, and corn oil for the culture of *S. carpocapsae* in cylindrical bottles agitated at 84 rpm and reported variations in the apparent viscosity of the broth, from 5 to ≈50 mPa·s, and characterized as slightly dilatant (n = 1.2 [−]) and moderately pseudoplastic (n = 0.6 [−]) at the beginning and the end of the process, respectively. Similarity, flow behavior index values n (1[−] to 0.5 [−]) were observed by Chavarría-Hernández et al. (2007) in cultures of *S. carpocapsae* conducted in 4-L airlift bioreactor. Whereas the massive death of adult nematodes causes a decrease in nematode biomass concentration (g/L) and the consistency index K (mPa·s^n) in one of three experiments. In both studies, it is argued that the rheological changes may be due to biomass concentration, as well as other undetermined factors that involve possible substances released by the decomposition of dead or disintegrated nematodes, mainly second-generation adults.

Recently, a fermentation broth composed of 2.3% (w/v) yeast extract, 1.25% (w/v) dehydrated egg yolk, 0.5% (w/v) NaCl, and 4% (v/v) canola oil, for the culture of *S. colombiense*, was rheologically characterized by Pérez-Campos et al. (2019) using an ARES G2 TA rheometer equipped with a concentric cylinder fixture. It is reported that the broth exhibits a Newtonian behavior (μ = 0.001 Pa·s) in the beginning, but after 48 hr of bacterial fermentation, the whole broth and its supernatant show a pseudoplastic behavior that remains during the 10-day process time. Considering the broth as an aqueous system, the change in rheological properties is attributed to the suspended particles (coagulated egg yolk, oil drops, and flexible cylindrical particles of different sizes depending on the development stage of nematodes) and the extracellular polysaccharide that is produced by *Xenorhabdus* or *Photorhabdus* (Amos et al., 2011; Drace & Darby, 2008; Jones et al., 2010). However, these premises must be confirmed with more evidence.

In general, it is assumed that the broth viscosity increases during the processing, which implies that the hydrodynamic resistance also increases. The mixing, increasing agitation, and/or aeration allows to overcome this resistance, but could affect the IJ-yields. Higher shear rates generated by agitation to overcome the hydrodynamic resistance deform the fluid elements and can be detrimental to nematodes because the transmitted shear and normal stresses can cause stress or physical damage, especially to fragile hermaphrodites of *H. bacteriophora* (Fife et al., 2004; Peters et al., 2017) and J1 stages of *S. carpocapsae* (Chavarría-Hernández et al., 2003). On the contrary, too low agitation rates are undesirable due to flocculation and aggregation caused, or even, by the growth of organisms in the wall reactor and agitator. Therefore, due to the contamination risk of the cultures associated with the extension of the processing time, it is a requirement to realize an adequate agitation of the broth for a uniform distribution of the medium ingredients and microorganisms (Masurekar, 2008; Yoo et al., 2001).

4.6 | Nematode tolerance to shear stress due to agitation

Pace et al. (1986) established that a speed rate at the tip of the impeller <0.3 m/s would avoid physical damage to adult nematodes. Then, Friedman et al. (1989) refuted the validity of Pace's procedure to determine this value and established that each nematode development stage is sensitive to rupture at different shear rates, suggesting that the gravid female stage is more sensitive to shear conditions.

However, knowledge of the mechanical properties of EPNs, the relationship between the stirring rate in bioreactors or hydrodynamic forces, and physical damage caused to the EPNs in the different developmental stages is still not available. One reason is that the fluid flow within a bioreactor implies shear and normal stress components that have so far not been isolated (Fife et al., 2004). In an experimental study, loss of viability of 85% of *S. carpocapsae* J1 stage has been observed when they are subjected to shear stress conditions, $\tau_{r\theta}$, 0.9–3.5 Pa, during a period of 80–100 min (Chavarría-Hernández et al., 2003).

The energy dissipation rate (ε) is a hydrodynamic parameter useful to characterize the physical damage in cell cultures involving mixing, assuming that all the viscous energy being dissipated by the fluid element will be fully realized by the body organism (Fife et al., 2004). Studying the flow of EPN suspensions through sudden contractions, the relative physical damage on IJ stages of *H. bacteriophora*, *H. megidis*, *S. carpocapsae*, and *S. glaseri* has been determined, which is associated with the energy dissipation rate that must be $<1 \times 10^8$ W/m^3 to maintain the integrity of EPNs (Fife, Derksen, Ozkan, & Grewal, 2003; Fife et al., 2004).

A proposed experimental strategy to study the damage to nematodes in submerged culture involves simulating the internal flow using computational methods of fluid dynamics (Joshi et al., 2011), for example, using the Ansys™ Fluent software to predict the distribution of energy dissipation rate in a bioreactor (Liu, Zhou, & Zhang, 2019). Then, the maximum ε could be calculated with the equation $\varepsilon_{max} = E \times N^3 \times D^2 \times \rho$ (Hu, Berdugo, & Chalmers, 2011), where N is the agitation speed (s^{-1}), D is the impeller diameter (m), ρ is the density (kg/m^3), and a dimensionless constant E that depends on (a) impeller type, (b) impeller diameter/vessel diameter, and (c) of-bottom distance/vessel diameter; some values have been reported for specific bioreactor configurations (Zhou & Kresta, 1996). For evaluation of physical damage, acetic acid or sodium chloride have been used as chemical stimulators to facilitate the determination of EPN viability; likewise, the image analysis obtained by bright field or scanning electron microscopy allows to determine rupture or deformation of EPNs (Fife et al., 2004; Viaene et al., 2010; Wilson, Pearce, & Shamlou, 2001).

4.7 | Oxygen demand by the bacteria–nematode complex, agitation, and aeration rate

In cultures of *H. megidis* in 5–10-L stirred tanks and 40% controlled saturation of DO, increasing aeration rates from 0.3 and 0.7 air-volumes per liquid-volume per minute (vvm) doubling the IJ production, and this could happen because of secondary metabolites that are negative for nematode survival were exported out of the liquid phase, which favors the reproduction (Strauch & Ehlers, 2000). In contrast, yield production of *S. carpocapsae* in an airlift bioreactor with external recirculation and working volume of 0.5 L follows a decreasing relationship at aeration rates of 0.05 and 0.15 vvm, suggesting that low airflow rates promote heterogeneous distributions of female and male adult nematodes in the vessel (Neves, Simões et al., 2001; Neves, Teixeira et al., 2001). In this sense, the dimensionless Reynolds number $Re = (D_H \nu \rho)/\eta_a$ was used as an index of hydrodynamic conditions within a pneumatically agitated bioreactor, where D_H is the hydraulic diameter, ν is the superficial gas velocity in two sections, ρ is the culture broth density, and η_a represents the apparent viscosity. The results suggested that if the conditions are laminar-type ($0.042[-] < Re < 6.4[-]$), the copulation process of steinernematids is favored because the IJ yield increase (Chavarría-Hernández et al., 2007).

Therefore, a premise of airlift bioreactors is to optimize the distribution of nematodes through proper configuration and aeration, to sustain sexual intercourse of steinernematids by creating low-speed circulation zones in liquid broth, where more females (because of higher density and mass) are concentrated, improving the mating opportunities with males that circulate through bioreactor (Chavarría-Hernández et al., 2007; Neves, Simões et al., 2001; Neves, Teixeira et al., 2001; Shapiro-Ilan et al., 2012). However, the reactor geometrical configuration (i.e., height-diameter ratio, internal and external) is a second factor that modifies *Re* number, where the higher the values, the higher the nematode production (Chavarría-Hernández et al., 2011).

The DO saturation percentage is a process parameter controlled through the operations of aeration and agitation (Belur et al., 2013). A challenge in liquid culture is to maintain the required DO concentrations (approx. 40% for IJs and 60% for adults) without increase the stirring speed too much since the hydrodynamics forces could negatively affect the nematode development stages sensitive to shear (Belur et al., 2013). In a bioreactor, the oxygen consumption rate during bacterial growth is higher than after inoculation of IJs, which enables a higher agitation rate to supply the required oxygen (Peters et al., 2017). But in aerobic processes, biomass production is commonly increasing in proportion to oxygen transfer rate and this, in turn, is proportionally increasing with the stirrer diameter, so, as a general rule, a 1-vvm gas flow is suggested so as not to accumulate carbon dioxide (Inman et al., 2012; Meier et al., 2016).

The low solubility of oxygen molecules in water (6–8 ppm) and the stagnant liquid film that is surrounding the gas bubble are the limiting steps for their delivery toward the microorganisms growing in culture broth (Garcia-Ochoa, Gomez, Santos, & Merchuk, 2010; Leite et al., 2016b; Pérez-Campos et al., 2019). The gas–liquid mass transfer coefficient, k_La (hr^{-1}), allows to account the effects of the operating variables on the efficiency in the provision of DO to biological specimens in pneumatically or mechanically agitated bioreactors (Belur et al., 2013; Chavarría-Hernández et al., 2007; Garcia-Ochoa & Gomez, 2009). Some authors point out that in bioreactor systems with both operations, the k_La coefficient is influenced mainly by the agitation rate compared to the aeration rate (Belur et al., 2013; Wang & Zhang, 2006). However, at high aeration rates in microbubble airlift bioreactor, no increase in k_La was observed when the agitation was between 150 and 200 rpm (Kim et al., 2014).

As can be seen in Figure 4, the highest reported yield productivity of *S. carpocapsae* nematode (250,000 IJs/ml) has been in an airlift bioreactor, in operating conditions involving k_La values $<0.007\ s^{-1}$ and *Re* number >9,000. In so much so that k_La values $>0.016\ s^{-1}$ appear in mechanically agitated bioreactors. Table 6 presents some correlations used to determine k_La in the production of EPNs in laboratory-scale bioreactors. Also, review articles describing various methods to determine this parameter in other systems can be found in the fermentation-processes literature (Aroniada et al., 2020; Garcia-Ochoa & Gomez, 2009; Garcia-Ochoa et al. 2010; Ranganathan & Sivaraman, 2011; Zhang et al., 2016).

It is well accepted that low k_La values should be achieved during the lag phase and the beginning of the exponential phase of the growth of symbiotic bacteria. However, Belur et al. (2013) report an opposite result in submerged culture of *P. luminescens*. During the lag phase and early exponential phase of growth in a 10-L-bioreactor at 200 rpm agitation and 1.44 vvm aeration, a higher k_La value of $39.5\ hr^{-1}$ was achieved to satisfy a higher specific oxygen uptake rate by symbiotic bacteria (Belur et al., 2013). In this experiment, if a high k_La value was associated with higher biomass concentration (>14 g/L), better conditions could have been established for the recovery and growth of EPNs (Chavarría-Hernández et al., 2007).

The dimensionless Damköhler number (*Da*) with the suggested modification of Çalik, Yilgör, Ayhan, and Demir (2004) can be applied to find the rate-limiting step of the bioprocess by comparing the rate among OTR_{max} of the system, determined by the product $E \times k_La \times C^*$, where *E* and C^* are the biological enhancement factor and the biomass saturation concentration in the liquid phase, respectively (Gomez, Santos, Alcon, & Garcia-Ochoa, 2006).

$$Da = \frac{OUR_{max}}{OTR_{max}}. \tag{9}$$

The maximum oxygen uptake rate (OUR_{max}) is given by $(m_{O_2} + Y_{OX} \times \mu_{max})C_X$, where m_{O_2} is the DO consumption coefficient, Y_{OX} is the macroscopic specific yield of oxygen, μ_{max} represents the specific growth rate, and C_X is the biomass concentration (Gomez et al., 2006). The criterion of this number is that if $Da > 1$, the rate of the biochemical reactions of consumption is more significant compared to the oxygen transfer rate, and if $Da \leq 1$, the rate of mass transport is higher compared to the rate of the biochemical reaction (Çalik, Yilgör, Ayhan, & Demir, 2004; Garcia-Ochoa et al., 2010; Gomez et al., 2006).

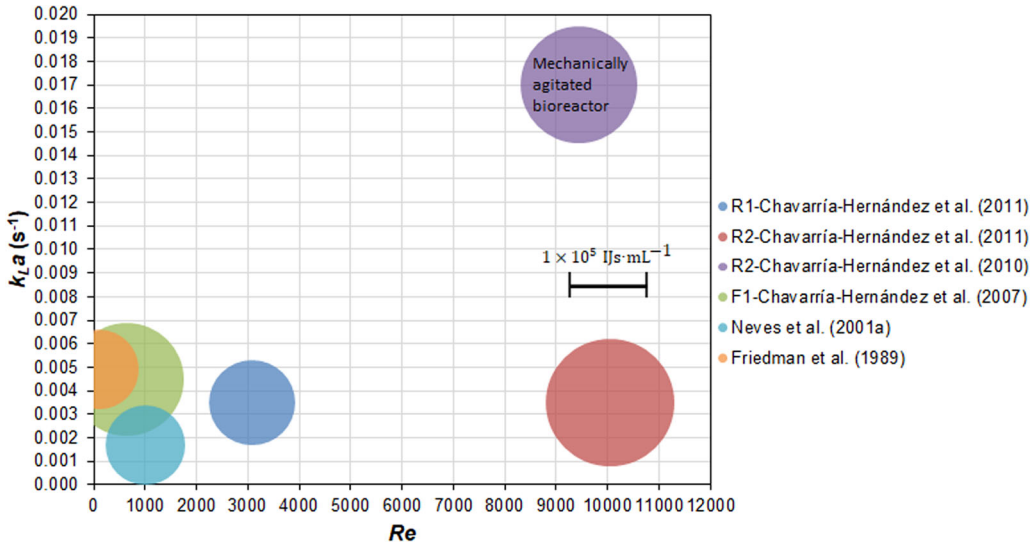

FIGURE 4 *Re* number (dimensionless), gas–liquid mass transfer coefficient (k_La, s^{-1}) and maximum final concentration (*C*, IJs/ml) reported or estimated by the authors of the present research review article, during in vitro production of *S. carpocapsae*, in airlift or mechanically agitated bioreactors. The circle size is proportional to the final concentration of EPNs. The scale bar represents 1×10^{-5} IJs/ml. R1 – Chavarría-Hernández et al. (2011): internal-loop airlift bioreactor, fermentation R1 using internal geometry h/d = 5.328 at 22°C during 16 days; R2 – Chavarría-Hernández et al. (2011): internal-loop airlift bioreactor, fermentation R2 using internal geometry h/d ratio = 2.894 at 22°C during 16 days; R2 – Chavarría-Hernández et al. (2010): fermentation R2 using internal draft tube and axial custom impeller at 22°C during 16 days; F1 – Chavarría-Hernández et al. (2007): internal-loop airlift bioreactor, fermentation F1 using a standard draft tube and whey-based culture medium at 22°C during 20 days; Neves, Simões et al. (2001): using an external-loop airlift bioreactor at 23°C, during 15 days; Friedman et al. (1989): using 125-ml flasks at 25°C and 150 rpm during 8 days. EPN, entomopathogenic nematode [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 6 Some mathematical relationships used to determine the gas–liquid mass transfer coefficient, k_La (hr^{-1}), in pneumatically or mechanically agitated bioreactors for EPN production at laboratory scale

System	k_La[a]		Reference
Internal-loop airlift bioreactor	$\frac{k_L a \times D_H}{\nu} = 2.95 \times 10^{-3} \times \left(\frac{D_{H\times\nu\times\rho}}{\eta_a}\right)^{0.37} \left(\frac{\nu^2 \times D_H \times \rho}{\sigma}\right)^{-0.13} \left(\frac{\nu}{\sqrt{g \times D_H}}\right)^{-0.31} \left(\frac{h}{D_H}\right)^{-0.5}$	(4)	Chavarría-Hernández et al. (2007)
Mechanically agitated airlift reactors	$\left[0.5013 - 0.0769\left(\frac{P}{V_L}\right)^{0.18}\right] v_{sg}^{(1.5732-0.1179N)}$	(5)	Chavarría-Hernández et al. (2010)
Internal-loop airlift bioreactor	$k_L a = 0.0343 \times v_{sg}^{0.524} \eta_a^{-0.255}$	(6)	Chavarría-Hernández et al. (2011)
Mechanically agitated bioreactors	$k_L a = \frac{OTR}{C^* - C_L}$	(7)	Belur et al. (2013)
Bioreactor with mechanical agitation and microaeration	$k_L at = \ln \frac{C_{L\infty} - C_L(0)}{C_{L\infty} - C_L(t)}$	(8)	Kim et al. (2014)

[a]Variables not previously declared: σ is the surface tension (N/m), *h* is the liquid height (m), *P* is the power consumption for mechanical agitation (W), v_{sg} is the superficial gas velocity (m/s), *N* is the rotation speed of the impeller (s^{-1}), V_L is the volume of culture broth (L or m^3), $C_{L\infty}$ is the DO concentration at saturation with air (mg/L), and $C_L(t)$ is the DO concentration at time *t* (mg/L). EPN, entomopathogenic nematode.

4.8 | IJ inoculation

Nematodes for inoculating the coculture broth can be obtained from in-vivo rearing or in vitro production, or fertilized eggs. The surface sterilization of IJ inoculum conducted by surface sterilization with sodium hypochlorite, thimerosal, or benzethonium chloride allows to exclude potential contaminants. Inman et al. (2012) report a detailed preparation of *H. bacteriophora* juvenile inoculum.

The IJ inoculum size and inoculation time are crucial process parameters for a thriving liquid culture. In particular, pH values of culture broth are well correlated with inoculation time and can be used for this purpose. Johnigk et al. (2004) found optimal conditions to supply the IJ inoculum of *H. bacteriophora* when pH exceeded the minimum value, during the stationary growth phase of the bacterial culture, because at this time, a high IJ recovery percentage is reached in hermaphrodites (40–50%) and the final yield productivity is maximized.

According to Leite et al. (2017), the yield productivity (121×10^3–178×10^3 nematodes/ml) of *S. feltiae* during liquid culture in Erlenmeyer flasks follows an increasing linear relationship with inoculum age (7–28 days). Based on this study, 7 days after inoculation, 65% of the IJs were formed as adults, and the best yield productivity was achieved using a 28-day old inoculum.

The recovery degree of nematode inoculum is a performance factor in the production process of heterorhabditids, and in the case of steinernematids, it is the maximization of mating between males and females (Shapiro-Ilan et al., 2012; Yoo et al. 2001). The performance of the process was analyzed by Peters et al. (2017) based on biological parameters associated with IJ recovery. The number of adult nematodes on the third day after inoculation of IJs is correlated with the final yield in liquid cultures of *S. feltiae* and *H. bacteriophora*, by the use of a two-parameter model. Now, it is known that final yield depends on optimal apparent fertility r (IJ per adult), which decreases linearly with the increase in the density of adults N (adults/ml) because there is competition for food, based on the following equation:

$$IJ = r\left(1 - \frac{N}{N_{crit}}\right)N, \tag{10}$$

where N_{crit} is the critical upper density of adults (adults/ml). This hypothesis agrees with the findings of Hirao et al. (2010), who observed that second- and third-generation adults of *S. feltiae* produced in monoxenic liquid culture do not contribute to the total IJ yield after 15-day postinoculation of nematodes. In contrast, IJ production of *S. carpocapsae* continued to increase 15 days after inoculation, because almost all offspring developed in IJs.

5 | CONCLUSION AND FUTURE PROSPECTS

The mass production of EPNs depends on induction of sexual reproduction; therefore, the selection of an efficient bacterial–nematode complex and the development of an adequate process of monoxenic liquid culture are focus research of great interest and significance to increase the yield production. In the last two decades, scientific and technological research conducted on nematode production in submerged monoxenic culture has focused on exploring the biological, physicochemical and technological factors to increase IJ productivity of the process, allowing to achieve yields of 460×10^3 IJs/ml and 252×10^3 IJs/ml, with *H. bacteriophora* and *S. carpocapsae*, respectively.

The technique of submerged monoxenic culture in small volumes has been simplified. The design of technology for the liquid culture of nematodes on a laboratory scale has been explored through adaptations to some equipment available in the market, as airlift bioreactors or mechanically agitated ones. However, available knowledge of industrial production of EPNs is marginal, limited to some process parameters and liquid media composition for the co-growth of the bacteria–nematode complex.

The culture broths are heterogeneous solid–liquid–gas systems; therefore, it is pertinent to conduct future research in the following disciplines of technological knowledge: bioprocess engineering, biosystem engineering, and mathematical and computational modeling of hydrodynamic behavior of liquid culture as a complex medium. The first two disciplines are used to optimize the process parameters for specific growth conditions of a particular bacteria–nematode complex. The last one is useful to construct mathematical approaches to improve the understanding of the process and to solve problems analytically, so as not to carry out unnecessary or expensive experiments on the system. On the other hand, the EPN quality has been marginally tested against target pest insects, so it remains as a less-explored field in science and technology for EPN mass production.

ACKNOWLEDGMENTS

The authors acknowledge Consejo Nacional de Ciencia y Tecnología (CONACyT), Mexico for the postdoctoral scholarship No. 741017 to Carlos I. Cortés-Martínez (CVU 178235), and Grants CB 2014, No. 238359; INFRA 2014, No. 230138, and INFRA 2016, No. 269805. The authors thank Dra. Adriana-Inés Rodríguez-Hernández for the critical review of this study.

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How to cite this article: Cortés-Martínez CI, Chavarría-Hernández N. Production of entomopathogenic nematodes in submerged monoxenic culture: A review. *Biotechnology and Bioengineering*. 2020;1–18. https://doi.org/10.1002/bit.27515