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# The Effects of Nutrient Concentration, Addition of Thickeners, and Agitation Speed on Liquid Fermentation of *Steinernema feltiae*

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*Abstract:* Entomopathogenic nematode production in liquid fermentation still requires improvements to maximize efficiency, yield, and nematode quality. Therefore, this study was aimed at developing a more suitable liquid medium for mass production of *Steinernema feltiae*, by assessing the effects of nutrient concentration, thickeners (primarily agar), and agitation speed on infective juvenile (IJ) yield. Base medium (BM) contained yeast extract (2.3%), egg yolk (1.25%), NaCl (0.5%), and corn oil (4%). All media were inoculated with *Xenorhabdus bovienii*, and 2 d later, with 2-d-old *S. feltiae* juveniles. For the nutrient concentration experiment, we evaluated the base medium versus a modified base medium containing all the components, but with 3× concentrations of yeast extract (6.9%), egg yolk (3.75%), and corn oil (12%). The nematodes and bacteria were cultured in 150-ml Erlenmeyer flasks containing 50 ml of liquid medium at (25°C) and 180 rpm on a rotary shaker incubator. To assess the effect of thickeners, IJs were inoculated in BM with agar (0.2%), carrageen (0.2%), and carboxymethyl cellulose (0.2% and 0.5%). The addition of 3× more nutrients relative to the BM resulted in a significantly lower yield of nematodes. For agar and agitation speed experiments, five levels of agar in the BM (0%, 0.2%, 0.4%, 0.6%, and 0.8% agar) and two agitation speeds (180 and 280 rpm) were evaluated for production. Increasing agitation speed from 180 to 280 rpm and higher levels of agar in the medium (> 0.2%) significantly increased the yield of bacteria. At the lower agitation speed, media amended with 0.4% and 0.6% agar produced higher nematode yields compared to media without agar. Media with 0.2% and 0.8% agar resulted in intermediate levels of nematode production. At the higher agitation speed, media supplemented with 0.8% agar resulted in the lowest yield of nematodes when compared to the other media tested. Results indicated that increasing nutrient concentration levels was detrimental to nematode production. Also, media containing agar (0.4% and 0.6%) increased nematode yields when cultures were grown at low agitation speed. When IJs were used as the inoculum, 0.2% agar also enhanced recovery and nematode yield at the higher agitation speed.

*Key words:* biocontrol, entomopathogenic nematode, in vitro production, liquid fermentation.

Entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae have a symbiotic association with bacteria, which makes them virulent against insects (Shapiro-Ilan and Gaugler, 2002). Thus, EPNs have been developed as biological control agents, especially to control soil dwelling insect pests (Shapiro-Ilan et al., 2014). These nematodes carry the bacteria in their gut. Once inside the insect host, the nematodes release their bacteria, which are primarily responsible for killing the host usually in 48 hr. The nematodes feed upon the proliferating bacteria and the digested insect tissues (Strauch and Ehlers, 1998). The nematodes complete 1 to 3 generations within the insect host, and then exit to search for new insect targets (Stock, 2015). The infective juvenile (IJ) stage is the only free-living stage and is adapted to be durable and survive in the soil environment until it can find a suitable insect host (Lewis and Clarke, 2012; Shapiro-Ilan et al., 2014).

More than 100 species of *Steinernema* and *Heterorhabditis* have been described, but only about one dozen have been commercialized for use in biocontrol (Lacey et al., 2015). *Steinernema feltiae* (Filipjev) is one of the most studied nematodes among the EPNs and it is used widely in biocontrol applications such as for suppression of fungus gnats, an important insect pest of ornamentals, nursery plants, and mushrooms (Jess et al., 2005; Tomalak et al., 2005).

EPNs have been mass produced using in vivo methods (by inoculating living insects), and in vitro methods, which use the bacteria culture as a food source for the nematodes (Ehlers, 2001; Shapiro-Ilan et al., 2014). In vitro methods include both solid and liquid fermentation. Because of its economy of scale, in vitro liquid culture is generally considered the preferred technology for large-scale commercial mass production of EPNs (Friedman 1990; Ehlers and Shapiro-Ilan, 2005). In vitro liquid production starts with shake-flask cultures and is scaled up to various sizes of bioreactors until reaching the desired level of commercial production. EPNs have been commercialized for nearly three decades, but their successful implementation in biological control has been limited. In part, this is due to noncompetitiveness of production costs compared with chemical insecticides as well as inconsistent nematode quality (Gaugler, 1997). Research on nematode liquid culture has focused on improving nematode production yield, quality, and costs. Maximum yields can be achieved through optimizing medium and culture conditions. Essential nutrients for nematode culture such as protein (Buecher et al., 1970; Yoo et al., 2001), lipids (Abu Hatab and Gaugler, 1999; Yoo et al., 2000), and various growth factors (Hieb and Rothstein, 1968; Hieb et al., 1970; Vanfleteren, 1974) have been studied. In addition, culture conditions such as temperature (Ehlers et al., 2000), inoculum size (Han, 1996), and aeration rate (Strauch and Ehlers,

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2000; Neves et al., 2001) have been optimized. However, in shake-flask production, no research has been reported to-date to address the effects of media that contain thickeners (such as agar) or high agitation speed (280 rpm) (which directly affects aeration). Therefore, this study was aimed at developing a more suitable liquid medium for mass production of *S. feltiae*, by assessing the effects of nutrient concentration, agar and other thickeners, and agitation speed on IJ yield. The experiments were conducted using shake flasks but the results may also be applicable for bioreactor conditions.

## Materials and Methods

Three experiments on in vitro production of *S. feltiae* were conducted using shake-flask cultures in a rotary shaker incubator, the first one to assess the effect of nutrient concentration, the second one to assess the effect of different concentrations of agar in the medium at two agitation speeds, and the third one to assess the effects of three thickeners standardized at one viscosity level.

*Bacterial isolation: Steinernema feltiae* (SN strain), originally isolated from soil in France, and stored in the nematode collection of USDA-ARS, Byron, GA, was used throughout our studies. The nematode's symbiotic bacteria, *Xenorhabdus bovienii* were isolated from infected hemolymph of last instar *Galleria mellonella* using a modified method by Akhurst (1980). The bacterial isolates were characterized on NBTA, and only bacterial isolates identified as purified phase I variants (Stock and Goodrich-Blair, 2012) were used. Bacteria stock cultures were established in tryptic soy broth (4%) + yeast extract (0.5%) medium, and frozen in 1 ml aliquots (with 15% glycerol) at −80°C (Kaya and Stock, 1997).

*Nematode axenization:* The nematodes were surface sterilized according to the procedures described by Lunau et al. (1993). Infective juveniles were surface sterilized using hyamine (0.1%) solution and rinsed three times. The IJs were concentrated in distilled water by precipitation, and the final suspension was spread onto nutrient agar medium (in 9-cm petri dishes) previously seeded with a culture of *X. bovienii*. Three days later, gravid females were collected from the petri dishes. The eggs were isolated from the nematodes and surface sterilized by alkaline lysis using 0.4 M NaOH (1.6% w/v) + NaOCl (1.1% w/v) followed by centrifugation at 5,000 *g* for 3 min. The eggs were rinsed three times in sterile water and transferred to 24-well plates containing 0.3 ml of TSB+ye liquid medium (4% tryptic-soy-broth + 0.5% yeast extract) in each well, for 2 d of incubation at 25°C. After incubation, the 2-d-old juveniles were used as nematode inoculum for the first two experiments. Infective juveniles were used as inoculum for the third experiment.

*Base medium:* For all the experiments, the base medium contained per liter: yeast extract (23 g), egg yolk (12.5 g), NaCl (5 g), and corn oil (40 g). This medium was selected as a high-yielding composition for production of steinernematids in a previous study conducted by Chavarría-Hernández and Torre (2001) for cultures starting from IJs.

*Experiment 1—Effect of nutrient concentration:* The study consisted of two treatments: the base medium and a modified base medium containing three times higher concentrations of yeast extract (69 g), egg yolk (37.5 g), and corn oil (120 g) per liter. Both base medium and modified base medium also included 0.2% agar. Nine replicates were established for each treatment, with each replication consisting of a 150-ml Erlenmeyer flask containing 50 ml of liquid medium. The media were inoculated with the bacteria *X. bovienii* previously grown in TSB+ye medium by adding 1 ml ($5 \times 10^9$ cells) of culture to each flask. The production flasks were incubated in a rotary shaker incubator (G 25; New Brunswick Scientific, Edison, NJ) at 180 rpm and 25°C for 2 d. After 2 d, the bacterial suspensions were inoculated with nematodes by adding 5,000, 2-d-old juveniles of *S. feltiae* (suspended in 0.3 ml of TSB+ye medium) to each flask (final concentration = 100 juveniles/ml).

The nematode–bacteria cultures were incubated in the rotary shaker incubator for 4 wk before counting nematode populations (IJs and other stages). For nematode counts, 1 ml of sample was taken from each flask and diluted 1:500 in water in a 1,000-ml beaker. The nematode suspension was stirred while taking a 2 ml sample for counting nematodes in a nematode counting chamber Peter slide using a stereomicroscope (16×). Counts were repeated twice. The entire experiment was repeated once in time (thus two complete trials).

*Experiment 2—Effect of agar and agitation speed:* This experiment consisted of 10 treatments and was conducted as a factorial with agar and agitation speed as the main factors. There were five levels of medium viscosity, which were developed based on the addition of agar to the medium at 0%, 0.2%, 0.4%, 0.6%, and 0.8% of the total volume. Two agitation speeds were tested: 180 and 280 rpm. Three replicates were established for each treatment, with each replication consisting of 50 ml of liquid medium in a 150-ml Erlenmeyer flask. The media were inoculated with *X. bovienii* previously grown in TSB+ye medium by adding 1 ml ($5 \times 10^9$ cells) of culture to each flask. The production flasks were incubated in a rotary shaker incubator (G 25; New Brunswick Scientific) at 180 rpm and 25°C for 2 d. After 2 d, the bacterial suspensions were inoculated with nematodes by adding 5,000, 2-d-old juveniles of *S. feltiae* (suspended in 0.3 ml of TSB+ye medium) to each flask (final concentration = 100 juveniles/ml).

The nematode production cultures were incubated in a rotary shaker incubator at 180 rpm (C24; New

Brunswick Scientific) and 280 rpm (Innova 4230; New Brunswick Scientific) for 4 wk at 25°C. Both incubators had the same orbital agitation. Culture evaluations were conducted 2 d after bacteria inoculation by counting bacteria cells, and weekly by counting bacteria cells and nematode populations. For the bacteria count, 25 µl of sample was taken from each flask and diluted in 40 ml water inside a centrifuge tube (50 ml). After the tubes were closed and vortexed, each bacterial suspension was counted in a Neubauer hemocytometer (Hausser Scientific, Horsham, PA) using a compound microscope (400×). For nematode counts, the methodology was the same as described previously. The entire experiment was repeated once in time (two trials).

*Experiment 3—Effect of thickeners:* The third experiment was conducted to assess the effects of three thickeners standardized at one viscosity level. This experiment consisted of five treatments: base medium; base medium + 0.2% agar; base medium + 0.2% carrageen (Iota Carrageenan; Ciao Imports, Miramar, FL); base medium + 0.2% carboxymethyl cellulose (CMC); and base medium + CMC 0.5%. Treatments were standardized based on both viscosity and concentration. The concentrations of 0.2% for carrageen and 0.5% for CMC were selected to provide a viscosity similar to 0.2% agar. Viscosity was measured based on the time the liquid flows through an orifice. All the three thickeners showed the same time (16 sec) to flow down from a 10-ml pipet, through 1-mm aperture tip. The flow times were assessed using the polymers/thickeners mixture with only water as diluent. The main purpose was to assess the effect of the biopolymers and compare them with agar, even though their viscosities were not exactly the same. The concentration of 0.2% CMC was also added so that all three thickeners would also be compared at the same concentration. Three replications were established for each treatment, with each replication consisting of 50 ml of liquid medium in a 150-ml Erlenmeyer flask. The media were inoculated with the bacteria *X. bovienii* previously grown in TSB+ye medium, by adding 1 ml ($5 \times 10^9$ cells) of culture to each flask. The flasks were incubated in an orbital shaker (Innova 4300 Incubator Shaker; New Brunswick Scientific) at 280 rpm and 25°C for 2 d. After this period, each flask was inoculated with IJs, by adding 2 ml of a 4-wk-old nematode culture in each flask, resulting in a final concentration 5,000 IJs per milliliter of medium.

Cultures were evaluated 2 d after bacterial inoculation by counting bacteria cells, 2 d after nematode inoculation by assessing nematode recovery, and 2 wk after nematode inoculation by counting nematode populations. For the bacterial counts, 25 µl of sample was taken from each flask and diluted in 40 ml water inside a centrifuge tube (50 ml). After the tubes were closed and vortexed, each bacterial suspension was counted in a Neubauer chamber using a compound microscope (400×).

For assessing nematode recovery, 100 µl of sample was taken from each flask and diluted in 20 ml water inside a centrifuge tube (50 ml). After the tubes were closed and vortexed, the nematode suspension was observed in a nematode counting chamber Peter slide using a stereomicroscope (16×), and the recovery was assessed. IJ recovery was assessed according to Hirao and Ehlers (2009) and represented the sum of the egg-juvenile 3 (J3), J4, and adult nematodes relative to the total number of the nematode population.

For nematode counts, the methodology was the same as described previously. The entire experiment was repeated once in time (two trials).

*Statistical analyses:* In each experiment, the trial × treatment interaction was assessed using analysis of variance (ANOVA); if no interaction was detected, then data were pooled across trials (otherwise the trials, i.e., repeated experiments, were analyzed separately). In the nutrient concentration experiment (Experiment 1), differences between media were detected using a *t* test. In Experiment 2 (effect of agar and rotation speed) and Experiment 3 (effects of different thickeners), treatment effects were detected using ANOVA. If the *F* value was significant, then treatment differences were further elucidated with Tukey's test. Additionally, in the factorial experiment (Experiment 2), if the effects of agar and rotation speed were found to act independently (no interaction detected between them), then the analysis focused solely on these main effects, and simple effects were not elucidated further (Cochran and Cox, 1957). Numerical data (nematode or bacteria yield) were square-root transformed prior to analysis, and percentage data (recovery) were arcsine transformed (arcsine of the square root) (Southwood, 1978); nontransformed means are presented in the results section. All statistical comparisons were done using SAS version 9.0 software, with a $P$ value $\leq 0.05$ indicating significance.

## Results

*Effect of nutrient concentration:* For the nutrient concentration experiments, an interaction between trial and concentration was detected ($F = 17.09$; df = 1, 32; $P = 0.0002$), and thus the two trials were analyzed separately. However, in both trials the addition of three times more nutrients relative to the base medium resulted in a significantly lower yield ($t = 4.31$; df = 16, $P = 0.0005$ for Trial 1 and $t = 2.51$; df = 16, $P = 0.0233$ for Trial 2) (Fig. 1).

*Effect of agar and agitation speed:* For the agar and agitation speed experiments, the analyses of bacterial yields showed no interaction between agitation speed × agar ($F = 1.72$; df = 4, 44; $P = 0.1632$) or between trial × agitation speed ($F = 3.93$; df = 1, 44; $P = 0.0536$) but showed an interaction between trial × agar ($F = 6.54$; df = 4, 44; $P = 0.0003$). The increased agitation speed from

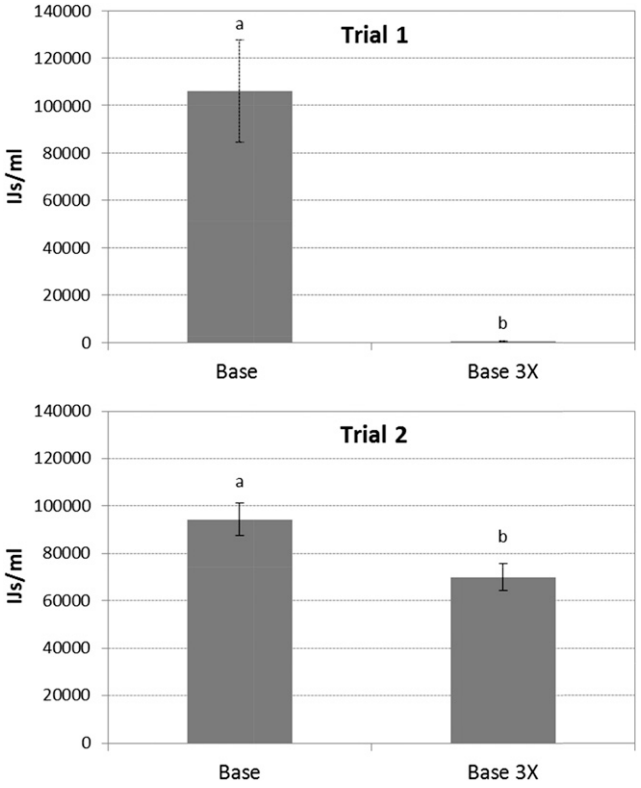


FIG. 1. Yields of *Steinernema feltiae* 28 d after nematode inoculation in liquid medium (Base) consisting of egg yolk (1.25%), yeast extract (2.3%), NaCl (0.5%), and corn oil (4%), and in the modified medium (Base 3×) containing a three times higher concentration of egg yolk and yeast extract. Both media also included 0.2% agar. The cultures were incubated at 180 rpm. Different letters above bars indicate statistical differences (*t* test; $P < 0.05$).

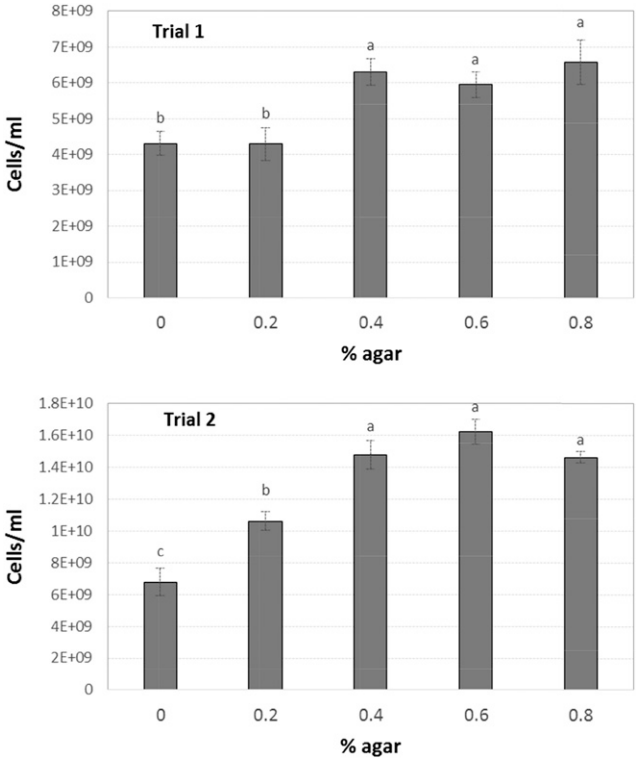


FIG. 3. Yields of *Xenorhabdus bovienii* cells 2 d post bacteria inoculation in the base liquid medium containing various concentrations of agar. Yields were averaged across two agitation speeds (180 and 280 rpm). Different letters above bars indicate statistical differences (Tukey's test; $P < 0.05$).

180 to 280 rpm caused an increase in bacterial cell count ($F = 21.46$; df = 1, 44; $P < 0.0001$) (Fig. 2). Also, in both trials, higher levels of agar in the medium (>0.2%) resulted in higher yields of bacteria ($F_{\text{trial 1}} = 12.4$; $P < 0.0001$; $F_{\text{trial 2}} = 31.35$; $P < 0.0001$; df = 4, 24 for both trials), with no difference detected among agar levels 0.4% to 0.8% (Fig. 3).

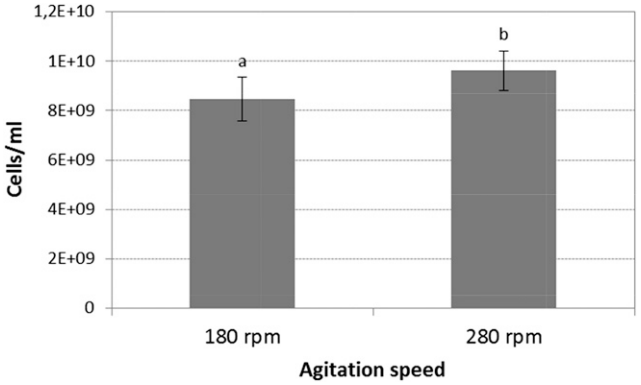


FIG. 2. Yields of *Xenorhabdus bovienii* 2 d post bacteria inoculation in liquid media. Yields were averaged across 0%, 0.2%, 0.4%, 0.6%, and 0.8% levels of agar. The cultures were incubated at 180 or 280 rpm. Different letters above bars indicate statistical differences (Tukey's test; $P < 0.05$).

For the assessment of nematode yields, no interaction was detected for trial × speed ($F = 2.73$; df = 1, 44; $P = 0.1059$) and trial × agar ($F = 1.71$; df = 4, 44; $P = 0.1654$) yet an interaction between main effects was detected ($F = 9.23$; df = 4, 44; $P < 0.0001$). The effects of agitation speed (180 and 280 rpm) differed when compared at different agar concentrations ($F_{0\%} = 20.53$, $P = 0.0014$; $F_{0.2\%} = 10.98$, $P = 0.009$; $F_{0.4\%} = 5.13$, $P = 0.0498$; $F_{0.6\%} = 6.52$, $P = 0.031$; $F_{0.8\%} = 9.33$, $P = 0.0137$; df = 1, 9 for all tests) (Fig. 4).

At an agitation speed of 180 rpm, 0.4% and 0.6% agar supported higher nematode yields compared to media with no agar, and media containing 0.2% and 0.8% agar resulted in intermediate levels of nematode production ($F = 4.41$; df = 4, 24; $P = 0.0082$) (Fig. 4). At the agitation speed of 280 rpm, media containing 0.8% agar supported lower yields of nematodes compared to all other agar levels tested ($F = 8.35$; df = 4, 24; $P < 0.0002$).

For all the cultures incubated at agitation speeds of 180 and 280 rpm (Fig. 5), the bacteria grew vigorously during the first week but not in the second week during nematode exponential growth, indicating that the bacteria might have been growing fast but being eaten even faster. By 20 d post inoculation, IJs were formed, becoming the primary stage found in the culture (> 98%).

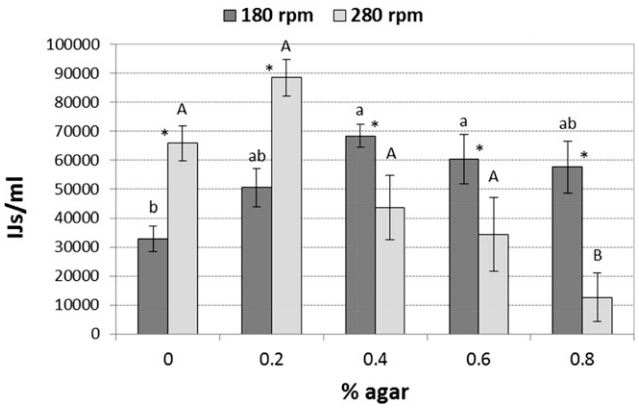


FIG. 4. Yields of *Steinernema feltiae* 28 d post nematode inoculation in the base liquid medium with different concentrations of agar. The cultures were incubated at 180 and 280 rpm. Different upper and lower case letters above bars indicate statistical differences within each agitation speed (Tukey's test; $P < 0.05$). *Statistical difference between 180 and 280 rpm for the particular agar level ($P < 0.01$).

*Effect of thickeners:* The analyses of bacterial yields, nematode recovery, and nematode yields showed no interaction between trial and thickener ($F = 1.34$; df = 4, 20; $P = 0.2907$ for bacteria; $F = 1.88$; df = 4, 20; $P = 0.1529$ for recovery; and $F = 1.09$; df = 4, 20; $P = 0.39$ for yield). Agar was the only thickener that improved bacteria growth, nematode recovery, and nematode yield compared with the control (base media without any thickener) ($F = 8.9$; df = 4, 20; $P = 0.0003$ for bacteria; $F = 11.83$; df = 4, 20; $P < 0.0001$ for recovery; and $F = 9.71$; df = 4, 20; $P = 0.0002$ for yield) (Fig. 6).

## DISCUSSION

The potential for efficiently producing EPNs using in vitro culture technology has been recognized since 1930 when Rudolf Glaser developed the first artificial culture method. Research on liquid culture production of EPNs has focused on improving yield, nematode quality, and production costs by optimizing medium and culture conditions (Cho et al., 2011). However, no study has been conducted to assess the effects of media with agar and high agitation speed (280 rpm) on nematode production.

Our results indicated that increasing nutrient concentration levels was detrimental to nematode production. Similar results were obtained by Yoo et al. (2001) using *Heterorhabditis bacteriophora*, with 3× nutrient concentrations decreasing IJ yields by more than 50%. According to Gil et al. (2002), glucose concentrations of 75 mg/ml or higher severely inhibited *H. bacteriophora* production and bacterial growth. Nutrients, including carbon, nitrogen, and mineral sources, can suppress metabolism of organisms when present at excessive concentrations (Gil et al., 2002; Leite et al., 2003). The concentration of the media components could create high osmotic pressure, which could significantly reduce bacterial growth. The reason for

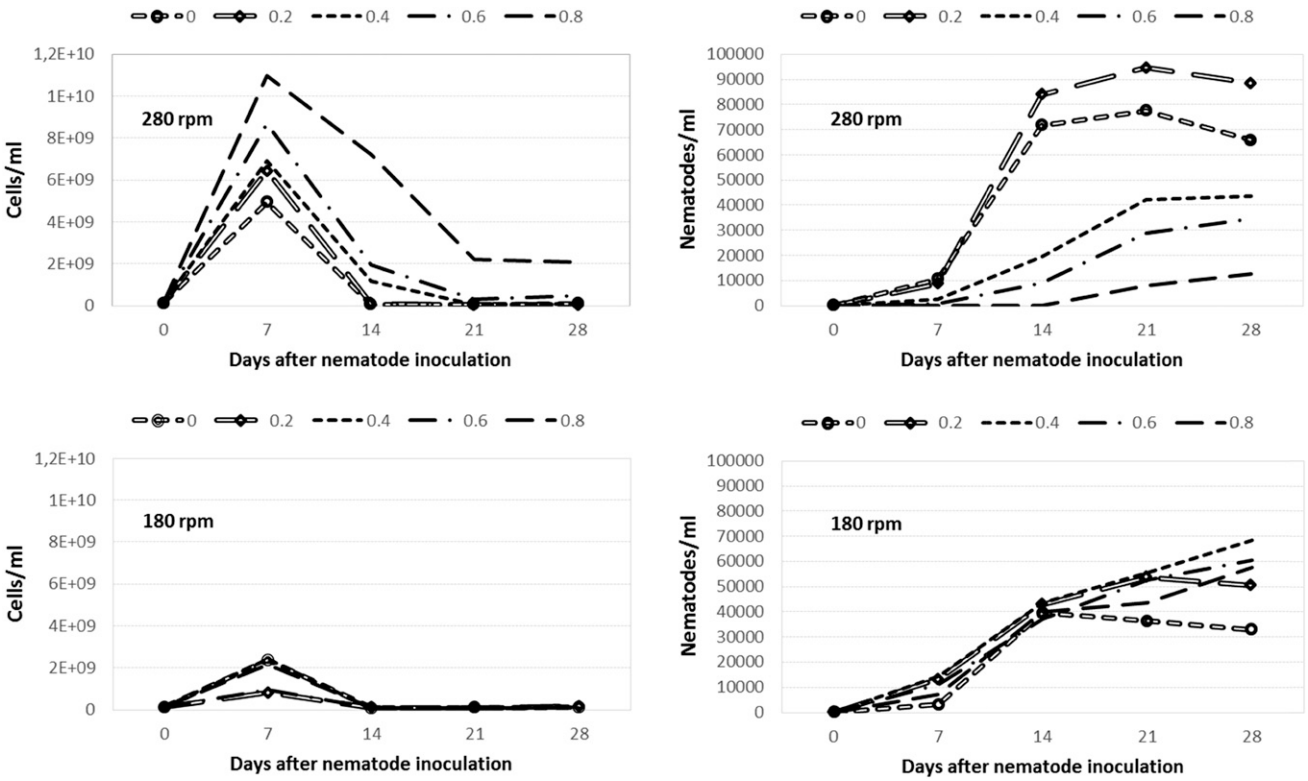


FIG. 5. Growth of *Steinernema feltiae* and its symbiotic bacteria *Xenorhabdus bovienii* in liquid media with different concentrations (%) of agar and incubated in a rotary shaker incubator at 180 or 280 rpm and 25°C.

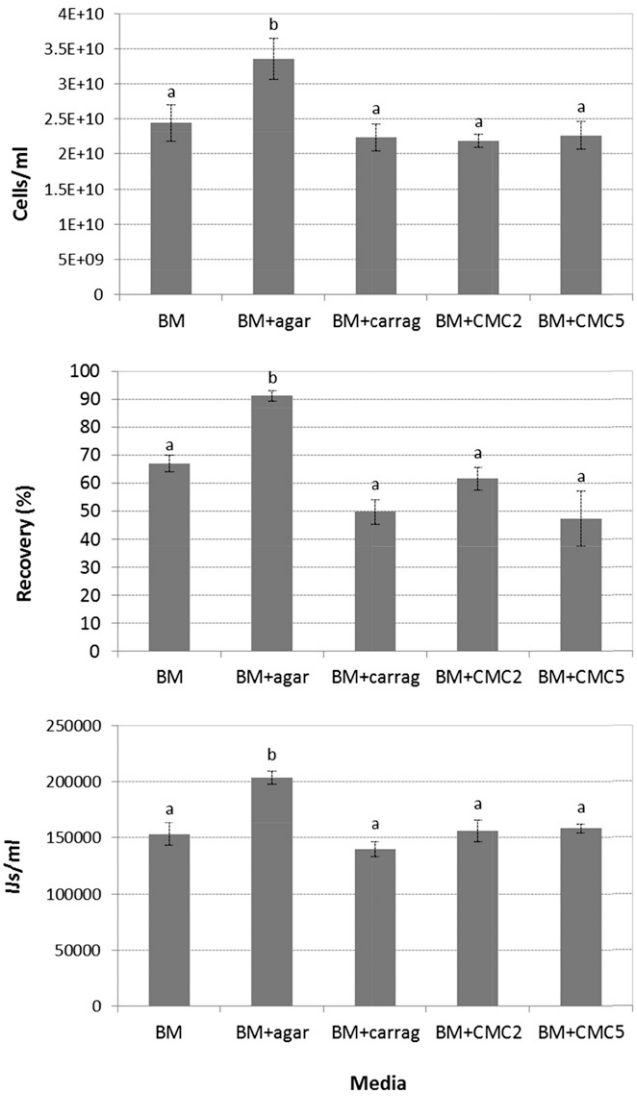


FIG. 6. (A) Yields of *Xenorhabdus bovienii* cells 2 d post bacteria inoculation (cells/ml), (B) percentage recovery of *Steinernema feltiae* 2 d post nematode inoculation, and (C) nematode infective juvenile (IJ) yields 28 d post nematode inoculation in base liquid medium (BM); BM + 0.2% agar (BM+agar); BM + 0.2% carrageen (BM+carrag); BM + 0.2% carboxymethyl cellulose (BM+CMC2); and BM + 0.5% carboxymethyl cellulose (BM+CMC5). The cultures were rotated at 280 rpm. Different letters above bars indicate statistical differences (Tukey's test; $P < 0.05$).

differences in yield obtained for the modified media in trials 1 and 2 is not clear, but surely is related to the detrimental effects of the modified medium, which made nematode growth more unstable. The same methodology was used for both trials, but any slight variation in an unsuitable medium may result in substantial differences in final yield.

Our study also showed that media containing agar resulted in increased nematode yields when cultures were grown at a low agitation speed, and that a high agitation speed increased nematode production for media without agar and with 0.2% agar. Two possible reasons for the positive effect of agar at lower agitation speeds may be an improvement of oil and air distribution in the medium. The lipids in flasks with agar appeared to be better dispersed than those without agar. In media without agar, the oil was less miscible, tending to concentrate on the surface of the liquid medium, especially at lower agitation speeds (personal observation). An improved distribution of lipids may make them more available to the nematodes and/or bacteria. Indeed, Hassane et al. (2012) used agar at 0.2% to obtain a homogenous distribution of essential oils in culture media.

At the lower agitation rate, agar may have also improved nematode growth by increasing dissolved oxygen. Previous liquid culture studies showed that increasing medium viscosity increased dissolved oxygen levels (Giese et al., 2013; Mascarin et al., 2015). Giese et al. (2013) demonstrated that high-viscosity media tend to adhere to the flask wall, thus increasing oxygen transfer by increasing the surface area of the medium. In our case, it may be that agar's role in integration of the oil phase into the liquid medium may also have improved oxygen transfer by increasing exposure of the medium to air. Thus, the same property of agar that increased oil distribution may have also increased oxygen transfer. At the higher agitation rate, agar was of less importance because we provided higher aeration and enough oxygen for the nematode growth in the media.

Agar may also be useful for nematode production by indirectly increasing the bacterial concentration. Agar has been mentioned as a source of carbon that supports growth of microorganisms (Ryan, 1950; Hankinson, 1974; Fiddy and Trinci, 1975; Payton, 1976). However, in our study, rather than being a direct source of carbon for the bacteria, agar may have benefited bacterial growth by increasing the miscibility of the oil and, consequently, improving lipid availability as a carbon source.

In contrast to the positive outcome of agar used at low agitation speed, the highest agar concentration combined with the higher agitation speed was detrimental. The reduction of nematode yields in media with the highest concentration of agar (0.8%) may have been due to reasons not related to the bacterial yield or oxygen availability. The bacterial yields remained high in media containing 0.8% agar regardless of the agitation speed. Some reaction between 0.8% agar and 280 rpm, such as friction, may have hampered nematode growth. Viscosity is caused by friction within the liquid, and too much friction may be detrimental to the nematodes.

There are a few studies addressing the effect of aeration on production of EPNs, most of them involving production inside the fermenter (Strauch and Ehlers, 2000; Kim et al., 2014). Oxygen transfer is the central problem for the bacterial symbiont in commercial bioreactors (Gaugler and Han, 2002). Regarding the nematode oxygen demands, studies have addressed the importance of this parameter (Shapiro-Ilan et al., 2012; Shapiro-Ilan et al., 2014), and Chavarria-Hernández et al. (2014) found specific demands ($qO_2$ values) of 1.92, 5.48, 0.48, 0.28, and 0.0014 [$10^{-1}$ $mmolO_2$/

(g nematode-wet base h)] for the J1, J2, J3, J4, and adult stages of *Steinernema carpocapsae*, respectively. Oxygen is poorly soluble in water resulting in the need for stirring impellers, gas-injecting spargers, and internal loops to increase transfer. The result is agitation, which resolves the bacterial oxygen requirements but conversely triggers shear force detrimental to the nematodes (Gaugler and Han, 2002). Our results indicate that agar improves nematode yield inside flasks kept at lower agitation speeds, which suggests the need for studies to assess the effect of agar on nematode production inside the bioreactor.

Interestingly, at the higher agitation speed, media containing 0.2% agar enhanced the yield but only when IJs were used as inoculum. This increase may have been due to improved IJ recovery (Fig. 6). Recovery is important to improve synchronism of copulation and final yield (Strauch and Ehlers, 1998; Jeffke et al., 2000; Shapiro-Ilan et al., 2012). The reason for the improved recovery in the flasks with agar might be due to improved distribution of oil and air as mentioned for the nematode growth. However, the question must be raised as to why the other two thickeners did not improve recovery similar to agar. Apparently, agar may have some beneficial properties that the other two thickeners do not have.

First stage juveniles of *S. feltiae* were used as inocula for the liquid media evaluated in our first two experiments; the inoculum was added at 100 juveniles/ml of medium. This concentration is 50-fold lower compared with the 5,000 IJs/ml of inoculum used by Hirao and Ehlers (2009). Inoculum based on a standard stage juveniles, such as on first stage juveniles, may favor synchronism of copulation and provide better growth of nematodes, which has been observed for inoculation with IJs (Strauch and Ehlers, 1998; Jeffke et al., 2000; Shapiro-Ilan et al., 2012). Indeed, we found the use of the first stage juveniles (at 100 nematodes/ml) to be convenient for standardization and effective in producing consistent yields and treatment differences. However, use of first stage juveniles in commercial mass production may be problematic relative to using IJs. Inoculum based on IJs has been successfully used for commercial production because high concentrations of IJs can be easily obtained and stored, increasing nematode yields and advancing harvest in 2 wk post inoculation (Strauch and Ehlers, 1998; Jeffke et al., 2000; Yoo et al., 2000; Hirao and Ehlers, 2009; Shapiro-Ilan et al., 2012).

In conclusion, the use of 0.2% agar in liquid media might be economically feasible and may be useful for inoculum, especially if the shaker does not provide high rotation speeds. Also, our study shows that high nutrient concentration may be harmful for the nematode, that agar may improve nematode growth, especially at lower agitation speeds, and that high agitation speeds improve nematode yield.

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