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Monoxenic production of the entomopathogenic nematode *Steinernema carpocapsae* using culture media containing agave juice (aguamiel) from Mexican maguey-pulquero (*Agave* spp). Effects of the contents of nitrogen, carbohydrates and fat on infective juvenile production

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Abstract The production of infective juvenile stages (IJ) of the entomopathogenic nematode *Steinernema carpocapsae* in the presence of its symbiotic bacterium *Xenorhabdus nematophilus* was carried out in orbitally agitated bottles. Four complex culture media (M1–M4) were used, containing from 8% to 28% (by vol.) agave juice (aguamiel) from Mexican maguey-pulquero (*Agave* spp) as the main carbohydrate source. After 20 days of fermentation, a maximum viable IJ concentration of 249,000 IJ/ml and an initial nematode population multiplication factor of ×620 were achieved when medium M4 was used (aguamiel concentration in this medium was 28% by vol.). M4 medium contained (w/v): 0.3% total nitrogen, 3.2% total carbohydrates and 3.0% total fat. According to the results obtained, total carbohydrates concentration appeared to be of great importance in obtaining high IJ concentrations.

Introduction

In recent years, several investigations concerning bioinsecticide production technologies have been conducted in view of the apparent negative ecological consequences due to the extensive use of chemical insecticides in agroindustry (Ehlers 2001; Samish and Glazer 2001). Among the bioinsecticides studied, where the role of *Bacillus thuringiensis* is notable (Lopez-y-Lopez et al. 2000), entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* (Ehlers 2001) are gaining attention in view of their success as biocontrollers of different coleopteran and lepidopteran insect pests (including borers and root weevils; Friedman et al. 1989).

The entomopathogenic nematodes are symbiotically associated with certain enterobacteria (Forst et al. 1997; Shapiro-Ilan and Gaugler 2002). *Steinernema* spp are associated with *Xenorhabdus* spp which exhibit two phenotypes, only one of which (the so-called phase I) is essential for effective killing of the insect host and effective nematode reproduction (Volgyi et al. 1998). Steinernematids have a life cycle that includes the egg, four juvenile stages (J1–J4) and the adult stage. Under certain conditions, the non-resistant J3 juvenile stage transforms into the resistant infective juvenile stage (IJ), which survives outside the insect host in the soil and has the ability to find and invade its prey, to continue its biological cycle (Friedman et al. 1989). The IJ is a particular developmental stage of the nematode that carries cells of the symbiotic bacterium within an intestinal vesicle (Vivas and Goodrich-Blair 2001).

Once an insect has been invaded by IJ, the symbiotic bacteria are released within the insect hemocoel. As a result, the insect dies within 24–48 h and conditions are appropriated for nematode growth and reproduction. Therefore, the IJ is of particular interest for field application and, according to different authors, the submerged monoxenic culture is the best technology for the mass production of IJ stages (Pace et al. 1986; Surrey and Davies 1996; Shapiro-Ilan and Gaugler 2002).

Nowadays, some companies produce entomopathogenic nematodes on a large scale (Ehlers 2001). Nonetheless, the processes involved are still empirical and researchers must improve the technology through the study of various aspects.

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Among these aspects, research concerning the study of culture medium is still scarce; and there are no reports on the use of by-products from agro-industry and non-conventional carbon sources from plants for IJ production.

The present work deals with the production of the entomopathogenic nematode *S. carpocapsae* in the presence of its symbiont *X. nematophilus*, using different culture media containing agave juice (called aguamiel) from Mexican maguey-pulquero (*Agave* spp) as an important carbon source.

In Mexico, aguamiel is traditionally used to manufacture pulque, an ancient pre-Hispanic naturally-fermented beverage (Guerrero-Guerrero 1985; Steinkraus 2002). Pulque production constituted a profitable industry in Mexico during the first 70 years of the twentieth century, but not today, as a result of the complex interaction of different economic, technological and social variables (Narro-Robles et al. 1992; Ramírez 1995). Among these, one can mention: (a) a permanent diminishing of pulque demand (especially since the 1960s) as a result of the notable growth of beer consumption, (b) the pulque price (nowadays, 1 l of traditional pulque is U.S. $ 0.26–0.43, depending on the producer) and (c) maguey depredation. Maguey plantations are being destroyed by certain people who either use some parts of the plants to prepare traditional dishes like barbacoa or look for exotic edible insects that live within the maguey plants during certain larval stages (i.e. chinicuiles, gusanos blancos). As a result, the culture of the maguey-pulquero plant has been discouraged and, as a matter of fact, there is an important decrease in maguey plantations. In view of the former information, there is concern at the present time to encourage the culture and rational use of the maguey-pulquero and its derived products.

The present report is part of research which intends to find out viable alternatives for the sustainable use of Mexican maguey-pulquero plants, particularly in the central part of Mexico (i.e. States of Mexico, Hidalgo, Puebla, Tlaxcala). Besides, some investigations have been done concerning the use of aguamiel as the main carbon source in fermentations other than pulque manufacturing (Sánchez-Marroquín et al. 1966; Sánchez-Marroquín et al. 1969).

Materials and methods

Specimens

Sanitized IJ of *S. carpocapsae* (Mexican strain; Lindegren et al. 1993; originally donated by Dr. R. Alatorre, Colegio de Postgraduados, Mexico) were maintained in 50-ml sterile-distilled water suspensions (300,000 IJ/ml) within cell culture flasks (culture area 150 cm^2, total volume 450 ml) at 4°C with aerating periods of 15 min every 15 days. *X. nematophilus* (phase I) was conserved in 2-ml vials (10^9 bacteria/vial) in 25% glycerol at −80°C. The symbiotic bacterium was isolated from NBTA culture plates (Akhurst 1980) streaked with hemolymph from last instar larvae of *Galleria mellonella* previously infected (24 h) with IJs of *S. carpocapsae* (Woodring and Kaya 1988).

Culture media

Six culture media were used, including TYB medium [Buecher and Popiel 1989; 3% (w/v) trypticase soy broth, 0.5% (w/v) yeast extract] and NBTA medium [Akhurst 1980; 2.3% (w/v) nutrient agar, 0.025% (w/v) bromothymol blue, 0.004% (w/v) triphenyl-tetrazolium choride (TTC)], *X. nematophilus* phase I absorbs bromothymol blue, producing dark blue colonies, whereas cells of *X. nematophilus* phase II do not absorb bromothymol blue, but reduce TTC and produce red colonies (Akhurst 1980). The four production/culture media tested (Table 1; M1–M4) contained different quantities of aguamiel from Mexican maguey-pulquero (*Agave* spp). Medium compositions were based on the medium P2 formulation (Chavarría-Hernández and de la Torre 2001). Aguamiel is a colourless and transparent liquid with a sweet taste, mainly constituted of maguey-pulquero sap. The components of aguamiel are water, sucrose, glucose, fructose, gums, certain proteins, minerals and vitamins, with a total carbohydrate concentration of

Table 1 Different culture media containing aguamiel from Mexican maguey-pulquero (*Agave* spp), used for the submerged culture of *S. carpocapsae* and *X. nematophilus*. All media contained 0.5% (w/v) NaCl and the pH was adjusted to pH 7.0 before autoclaving. Medium compositions are based on the medium P2 formulation (Chavarría-Hernández and de la Torre 2001). The contents of total nitrogen (N), carbohydrates (CH) and fat (F) were respectively determined on the basis of ingredient analyses by method 991.20 (AOAC 1999), the method of Dubois et al. (1956) and method 963.15 (AOAC 1999). Ingredients compositions are: (1) aguamiel: 0.07% N, 10.5% CH, 0% F, (2) yeast extract: 10% N, 12.5% CH, 0.3% F, (3) dried egg yolk: 7.6% N, 2.3% CH, 35.2% F, (4) corn oil: 0.08% N, 0.3% CH, 99.3% F

Culture medium	Aguamiel concentration (% v/v)	Yeast extract concentration (% w/v)	Dried egg yolk concentration (% w/v)	Corn oil concentration (% v/v)	Total nitrogen concentration (% w/v)	Total carbohydrates concentration (% w/v)	Total fat concentration (% w/v)
M1	8.2	0.4	0.5	5.3	0.09	0.94	5.42
M2	16.0	2.2	0.2	2.2	0.25	1.97	2.25
M3	22.2	0.2	1.7	0.7	0.16	2.39	1.21
M4	27.6	1.7	1.2	2.5	0.29	3.15	2.96

usually 10–20% (w/v; Mijangos-Santiago 1994; Martínez del Campo-Padilla 1999). The aguamiel was provided as refrigerated fresh-liquid by Ometeotl A.C., Tlaxcala, Mexico; and it was autoclaved in 2-l bottles (1 l of aguamiel per bottle) immediately after reception in order to avoid undesirable fermentation. Then, bottles containing sterile aguamiel were stored until medium preparation. The composition of the media in terms of the concentrations (w/v) of total nitrogen (N), total carbohydrates (CH) and total fat (F) were determined by mass balance, taking into account the corresponding contents of N, CH and F for each medium ingredient, that in turn were determined through method 991.20 (AOAC 1999), the method of Dubois et al. (1956) and method 963.15 (AOAC 1999), respectively. The composition of the four medium ingredients was (w/v): (1) aguamiel: 0.07% N, 10.5% CH, 0% F, (2) yeast extract (YE): 10% N, 12.5% CH, 0.3% F, (3) dried egg yolk (DEY): 7.6% N, 2.3% CH, 35.2% F and (4) corn oil (CO): 0.08% N, 0.3% CH, 99.3% F. The CO density was experimentally determined (0.921 g/ml). All production media contained 0.5% (w/v) NaCl and were pH-adjusted to pH 7.0 before autoclaving. As an example,

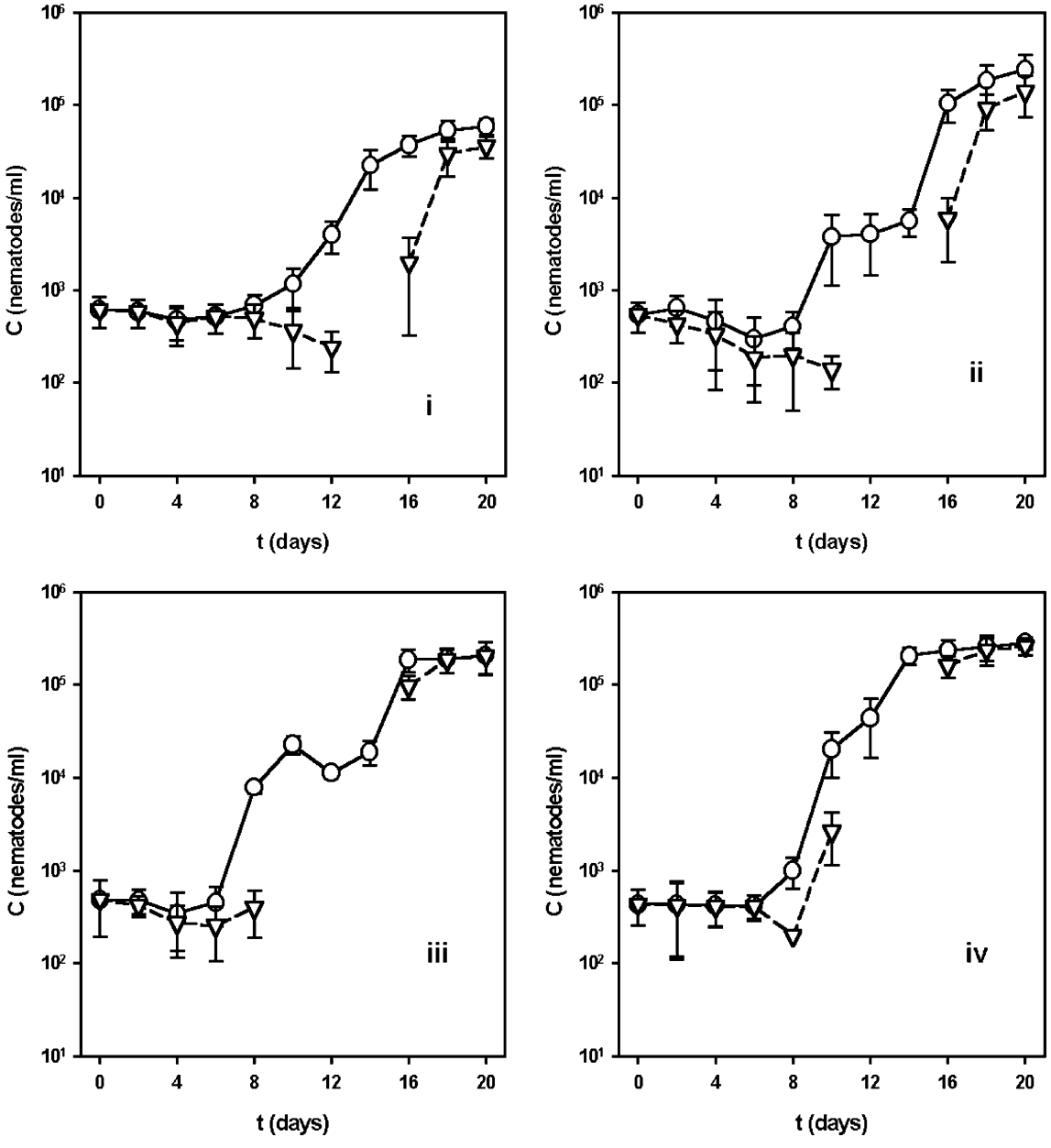

Fig. 1 Time-courses of both total viable nematode concentration (○, nematodes/ml), and IJ stage concentration (▽, IJ/ml) during the submerged culture of *S. carpocapsae* growing in the presence of *X. nematophilus* in orbitally agitated bottles. Four complex media (M1–M4) containing different proportions of aguamiel from Mexican maguey-pulquero (*Agave* spp) were tested: *i* M1 with 8.2% (v/v) aguamiel, *ii* M2 with 16% (v/v) aguamiel, *iii* M3 with 22.2% (v/v) aguamiel, *iv* M4 with 28% (v/v) aguamiel. Each plot represents three independent experiments, each with triplicate nematode counts per time-point (*t*)

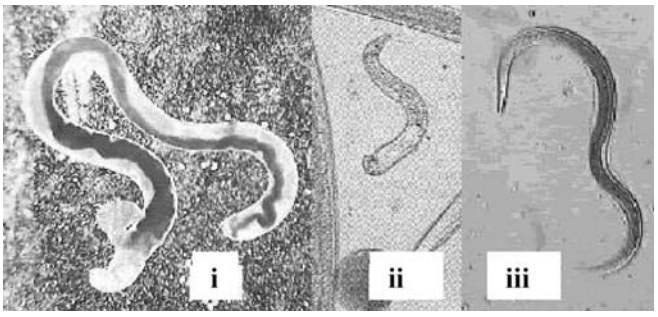

Fig. 2 Light microphotographs of *S. carpocapsae* from samples of submerged monoxenic cultures using medium M4 [28% (v/v) aguamiel from Mexican maguey-pulquero (*Agave* spp)]: *i* young first-generation adult female at day 6 (length 3,500 μm; magnification 40×), *ii* juvenile of the second stage (length 197 μm; magnification 400×), *iii* infective juvenile stage at day 20 (length 580 μm; magnification 100×)

the CH contents determination for medium M1 is shown (base: 100 ml of medium):

$$\begin{aligned} CH_{M1} &= CH_{aguamiel} + CH_{YE} + CH_{DEY} + CH_{CO} \\ &= [8.2\,ml_{aguamiel} \times 0.105\,g_{CH}/ml_{aguamiel}] + [0.4\,g_{YE} \\ &\quad \times 0.125\,g_{CH}/g_{YE}] + [0.5\,g_{DEY} \times 0.023\,g_{CH}/g_{DEY}] \\ &\quad + [5.3\,ml_{CO} \times 0.921\,g_{CO}/ml_{CO} \times 0.003\,g_{CH}/g_{CO}] \\ &= 0.937\,g_{CH} \end{aligned}$$

Monoxenic cultures

Cultures were carried out according to Chavarría-Hernández and de la Torre (2001) in orbitally agitated bioreactors. One *X. nematophilus* phase I vial was inoculated into 50 ml of sterile TYB contained in a 250 ml-Erlenmeyer flask and incubated at 29°C and 150 rpm until a concentration of 10^9 bacteria/ml was reached (24 h approx.). The bacterial phase was then checked by streaking a culture broth sample on NBTA plates. Thereafter, Erlenmeyer flasks (total volume 500 ml) containing 150 ml of the corresponding sterile M-medium (M1–M4) were inoculated with 5% (v/v) *X. nematophilus* in TYB culture broth and incubated at 29°C and 150 rpm during 48 h. Then, NBTA plates were streaked with broth samples to confirm the presence of *X. nematophilus* phase I.

Thereafter, culture broth was inoculated with IJ in water suspension to give approximately 500 IJ/ml (C_0) and, after mixing well, the broth was poured into three sterile plastic-top glass bottles (total volume 500 ml; i.d. 9 cm), pouring 50 ml into each one. Then, the M-culture bottles were incubated at 22°C and 150 rpm during 20 days, taking samples of 0.1 ml from each bottle every 2 days.

Determination of the viable nematode concentration

M-culture broth samples were diluted (1:10 to 1:1000), using an isotonic salt solution and 0.002% (w/v) methylene blue to stain nematodes. Concentrations of the total viable nematodes (C) and IJ stages were determined by counts in 0.1 ml of diluted samples under the light microscope (40×, 100×), in triplicate. The multiplication factor (C_{max}/C_0) was also determined, where C_{max} is the maximum C_{tot} and (C_{max}/C_0) indicates how many times the C_0 is folded through the whole process.

Results

All culture broth samples of *X. nematophilus* streaked onto NBTA plates developed almost 100% dark blue colonies, suggesting that *X. nematophilus* was mainly in phase I (Akhurst 1980).

Figure 1 presents the evolution of both the total viable nematode and IJ concentrations during the fermentations. Starting values of C_0 were 430–550 IJ/ml and the presence of first-generation adults in culture broths was first recorded at days 4–6 (Fig. 2i), depending on the medium used. The release of fertilized eggs by gravid first-generation females followed by hatching of new-generation nematodes (Fig. 2ii) occurred at days 6–8 and the nematode population consequently started to grow (Fig. 1).

In general, the recovery process of inoculated IJ finished at days 8–10, depending on the culture medium (Fig. 1; Ehlers et al. 1998). Thereafter, new-generation IJ were first observed at day 16, indicating the incipient appearance of non-favorable conditions to support further nematode population growth. Under such conditions, J3 stages transform into the resistant non-feeding IJ stage (Fig. 2iii; Woodring and Kaya 1988).

Table 2 Main results obtained during the submerged culture of *S. carpocapsae* and *X. nematophilus* in four culture media containing aguamiel from Mexican maguey-pulquero (*Agave* spp)

Culture medium	Initial nematode concentration, C_0 (nematodes/ml)	Maximum nematode concentration, C_{max} (nematodes/ml)	Maximum IJ concentration, $C_{IJ,max}$ (IJ/ml)	Percentage of infective juveniles at the end	Multiplication factor (C_{max}/C_0)
M1	553 (198)[a]	56,333 (10,214)[a]	33,000 (11,525)[a]	59	102
M2	489 (268)[a]	213,889 (94,536)[a]	116,666 (59,011)[a]	55	437
M3	442 (222)[a]	198,889 (51,217)[a]	196,666 (65,887)[a]	99	450
M4	428 (204)[a]	270,556 (43,151)[a]	249,444 (65,392)[a]	92	632

[a]Standard deviation $[= ((n\Sigma x^2 - (\Sigma x)^2)/(n(n-1)))^{1/2}]$

Table 3 Comparison of the maximum IJ stage concentrations reported by different groups concerning the mass production of entomopathogenic nematodes (*Steinernema* spp). Cultures were carried out in orbitally agitated flasks or bottles, unless otherwise stated. The contents of total N, CH and F were determined based on N, CH and F mass balances, considering the corresponding contributions of each ingredient as stated in Table 1. Other ingredient contributions in the referred reports were considered as follows: (1) de-fat soy flour (U.S. Department of Agriculture 2002): 8.2% N, 33.9% CH, 1.2% F, (2) ox kidney homogenate (considered as male cattle raw kidney; U.S. Department of Agriculture 2002): 2.8% N (crude protein basis, N × 6.25), 0.3% CH, 3.1% F, (3) EnlivPro (considered as 100% protein, crude protein basis: N×6.25): 15.4% N, 0% CH, 0% F, (4) cholesterol (considered as 100% fat): 0% N, 0% CH, 100% F

Nematode	Total nitrogen concentration (% w/v)	Total carbohydrates concentration (% w/v)	Total fat concentration (% w/v)	Maximum IJ stage concentration (IJ/ml)	Reference
[a]*S. feltiae*	0.38	0.15	0.3	40,000–70,000	Pace et al. (1986)
[b]*S. feltiae*	0.38	0.15	0.3	90,000	Pace et al. (1986)
[b]*S. carpocapsae*	0.34	0.83	2.31	38,000–60,000	Neves et al. (2001)
S. carpocapsae	0.34	0.83	2.31	14,700	Neves et al. (2001)
[c]*Neoaplectana carpocapsae*	0.58	1.06	5.16	102,000	Friedman et al. (1989)
[c]*N. carpocapsae*	0.38	0.76	5.38	100,000	Friedman et al. (1989)
S. feltiae	0.29	0.76	4.35	134,000	Chavarría-Hernández and de la Torre (2001; medium P1)
S. feltiae	0.32	0.31	4.42	190,000	Chavarría-Hernández and de la Torre (2001; medium P2)
S. carpocapsae	0.16	2.39	1.21	197,000	Present work (medium M3)
S. carpocapsae	0.29	3.15	2.96	249,000	Present work (medium M4)

[a]Mechanically agitated bioreactor
[b]Pneumatically agitated bioreactor
[c]*Neoaplectana* (≡ *Steinernema*)

At day 20 during fermentation, cultures achieved the highest nematode concentrations (C_{max}), from a minimum of 56,300 nematodes/ml (medium M1) to a maximum of 271,000 nematodes/ml (medium M4), with the proportion of nematodes in the IJ stage being 59% (i.e. 33,000 IJ/ml) and 92% (i.e. 249,000 IJ/ml), respectively (Table 2).

Considering that one of the main purposes of this fermentation is to achieve high concentrations of viable IJ (other purposes include achieving high contents of fat within IJ stages and high IJ virulence; Yang et al. 1997; Yoo et al. 2000), the results obtained in the present work are notable. Particularly, the achieved maximum IJ concentrations in M4 fermentations were higher than the corresponding ones in various previous reports dealing with the production of IJ stages of *Steinernema* spp (Table 3).

M1 fermentations rendered the minimum value of C_{max}/C_0 (120), whereas M4 fermentations rendered the maximum (C_{max}/C_0=620), which was 5.2 times the corresponding M1 value. Also, the value of C_{max}/C_0 for M4 was notably higher than other reported values concerning the IJ production of *Steinernema* spp, using similar production systems: C_{max}/C_0=107–204 (Friedman et al 1989) and C_{max}/C_0=228–372 (Chavarría-Hernández and de la Torre 2001).

M1 medium was the least satisfactory for the mass production of IJ stages of *S. carpocapsae* among all media studied. In contrast, the best results were obtained when medium M4 was used (Table 2).

The results obtained in the present work on nematode propagation could have a relationship with both the quality and the quantity of nutrients present in the media which were available for the specimens: the medium composition could affect in different ways the development of both *S. carpocapsae* and its symbiont *X. nematophilus*.

Discussion

The availability of proper culture media for the mass production of entomopathogenic nematodes through submerged monoxenic culture technology permits us to achieve high concentrations of IJs. Also, the quality of the produced IJs in terms of both viability and virulence should be good enough.

In the present work, different culture media containing aguamiel from Mexican maguey-pulquero (*Agave* spp) were evaluated for the mass production of IJ stages of *S. carpocapsae*, considering the achieved maximum viable-IJ concentration as the medium evaluation criterion.

The obtained results were outstanding, especially when medium M4 was used. In this case, a maximum concentration of 249,000 IJ/ml was achieved, notably higher than the corresponding values reported by other authors using similar production systems (Friedman et al 1989; Chavarría-Hernández and de la Torre 2001). Since the bioreactor design for large-scale IJ production is usually

different from the bottle format, our group is currently testing M4 medium in fermentation runs in a 4-l internal loop air-lift bioreactor.

For a fermentation process to be controlled, it is necessary to have information about the nutrient demands of the specimens, among other aspects. Therefore, in order to improve control of the *S. carpocapsae* IJ mass production process, one should have knowledge about the food requirements—carbohydrates, proteins, fats and vitamins, for instance—of the nematode and its symbiont. Unfortunately, the former aspects concerning the *S. carpocapsae*/*X. nematophilus* complex are practically unknown. Furthermore, the scant knowledge concerning nematodes is mainly in relation to *Caenorhabditis elegans* (Wright and Perry 2002).

The present work discusses an effort to determine the effect of medium composition on the production of IJ stages of *S. carpocapsae*. The medium composition is presented in terms of the concentrations of total nitrogen, total carbohydrates and total fat.

Table 3 presents the maximum IJ concentrations reported by different authors during the submerged culture of steinernematids in different bioreactor systems, using various complex-medium formulations. Also, it presents the results corresponding to the M3 and M4 media of the present investigation.

Among the different investigations cited in Table 3, the highest IJ concentration was obtained when medium M4 was used (the present work). Furthermore, the maximum IJ concentration using M4 medium was 249,000 IJ/ml, which is 1.31 times the corresponding value obtained using medium P2 (190,000 IJ/ml; Chavarría-Hernández and de la Torre 2001). These two media are similar to each other in their total nitrogen concentration ($N_{M4}/N_{P2} = 0.29/0.32 = 0.91$ times), but differ in the total carbohydrates ($F_{M4}/F_{P2} = 2.96/4.42 = 0.67$ times; $CH_{M4}/CH_{P2} = 3.15/0.31 = 10$ times). In fact, considerable differences in CH concentration are presented. Similar trends are observed when the results obtained using medium M3 (present work) are compared with those using medium P2 (Chavarría-Hernández and de la Torre 2001). Almost corresponding concentrations of total nitrogen ($N_{M3}/N_{P2} = 0.16/0.32 = 0.5$ times), total carbohydrates ($CH_{M3}/CH_{P2} = 2.39/0.31 = 7.71$ times) and total fat ($F_{M3}/F_{P2} = 1.21/4.42 = 0.27$ times). This means that an increase in the total carbohydrates of the culture medium used would improve the production of steinernematid IJ stages, although the concentrations of both the total nitrogen and total fat do not change or decrease (at least, within the experimental ranges discussed).

These results differ from those reported by Yoo et al. (2000) concerning the production of *H. bacteriophora*. According to those authors, the fat source—considering both nature and concentration—present in the culture medium is a major determinant for reaching high IJ concentrations, and the IJ quality also depends on the fat source used. Nevertheless, Yoo et al. (2000) propagated other nematode species and used culture media with sources of nitrogen, carbohydrates and fat—in both nature and concentration—which were different from those used in the present work. These differences should be taken into account when discussing the mentioned tendencies.

However, taking into account that generally both sources of nitrogen and fat are more expensive than the corresponding source of carbohydrate, it is possible that the results presented in this investigation could have implications concerning cost reduction in the large-scale production of *S. carpocapsae* IJ stages. In particular, the hypothesized cost reduction could be more attractive if the culture medium contains non-conventional carbohydrates sources like aguamiel from Mexican maguey-pulquero (*Agave* spp) which costs less than U.S. $ 0.11/l (traditional pulque manufacturers, personal communication).

Taking into account the actual Mexican maguey-pulquero situation that was presented in the Introduction, the use of aguamiel in certain parts of Mexico as the main carbohydrate source for the mass production of IJ stages would be a viable alternative and could re-activate the so-called Maguey-economy. This would contribute to a sustainable use of these *Agave* spp.

However, it is necessary to conduct studies to determine the specific nutrient requirements of the *S. carpocapsae*/*X. nematophilus* complex and how they would change during the fermentation process. Bioengineering studies are also necessary, particularly those concerning the effects of hydrodynamics, oxygen-transfer conditions and reaction kinetics on the production of steinernematid IJ stages. A reliable technology for the mass production of bioinsecticidal nematodes can be provided by the multidisciplinary efforts of research groups.

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