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MINI-REVIEW

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Mass production of entomopathogenic nematodes for plant protection

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Abstract Entomopathogenic nematodes of the genera *Heterorhabditis* and *Steinernema* are commercially used to control pest insects. They are symbiotically associated with bacteria of the genera *Photorhabdus* and *Xenorhabdus*, respectively, which are the major food source for the nematodes. The biology of the nematode–bacterium complex is described, a historical review of the development of in vitro cultivation techniques is given and the current use in agriculture is summarised. Cultures of the complex are pre-incubated with the symbiotic bacteria before the nematodes are inoculated. Whereas the inoculum preparation and preservation of bacterial stocks follow standard rules, nematodes need special treatment. Media development is mainly directed towards cost reduction, as the bacteria are able to metabolise a variety of protein sources to provide optimal conditions for nematode reproduction. The process technology is described, discussing the influence of bioreactor design and process parameters required to obtain high nematode yields. As two organisms are grown in one vessel and one of them is a multicellular organism, the population dynamics and symbiotic interactions need to be understood in order to improve process management. Major problems can originate from the delayed or slow development of the nematode inoculum and from phase variants of the symbiotic bacteria that have negative effects on nematode development and reproduction. Recent scientific progress has helped to understand the biological and technical parameters that influence the process, thus enabling transfer to an industrial scale. As a consequence, costs for nematode-based products could be significantly reduced.

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Taxonomy

Many insect antagonists are found within the phylum Nematoda, but only species within the genera *Steinernema* and *Heterorhabditis* (Rhabditida) have gained major importance as biocontrol agents in plant protection. Today, more than 30 species of these so-called entomopathogenic nematodes (EPN) have been described and many more will follow (Hominick et al. 1997). EPN are closely related to *Caenorhabditis elegans*, which is the current model organism for studying animal development and genetics (Riddle et al. 1997) and whose genome sequence has recently been completed. Unique to EPN is their close symbiotic association with bacteria of the genera *Xenorhabdus* and *Photorhabdus*. The symbionts belong to the Enterobacteriaceae, within the gamma subdivision of the purple bacteria (Ehlers et al. 1988). These bacto-helminthic complexes are used in the biological control of insects in cryptic environments (Ehlers 1996).

Life cycle

A special developmental stage within the life cycle of all rhabditid nematodes is the dauer juvenile (DJ). This term dauer (German for enduring) was introduced by Fuchs (1915) and describes a morphologically distinct juvenile, formed as a response to depleting food resources and adverse environmental conditions. The DJ is a free-living, third juvenile stage that is well adapted to long-term survival in the soil. The DJ is the infective stage that carries 200–2,000 cells of its symbiont in the anterior part of its intestine (Endo and Nickle 1994). It invades the insect host through natural openings (mouth, anus, tracheae) or directly through the cuticle (Peters and Ehlers 1997). In the haemolymph, the nematodes encounter optimal conditions for reproduction. They respond to yet unknown signals (Strauch and Ehlers 1998), which induce the exit from the developmentally arrested DJ stage. Pharynx, digestive tract and excretory metabolism are activated.

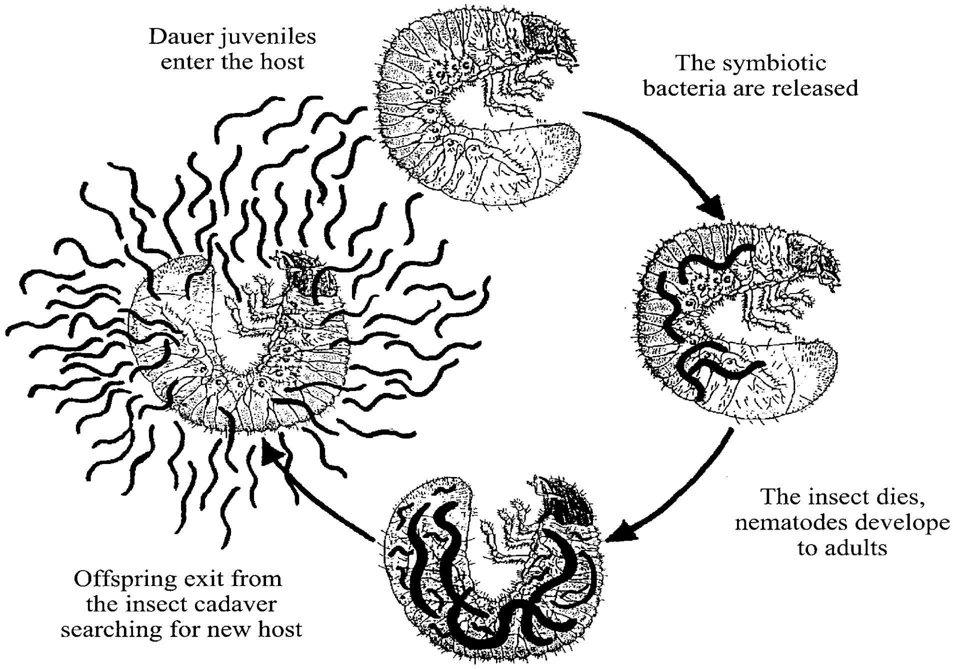

Fig. 1 Life cycle of entomopathogenic nematodes in infected larva of a scarabaeid beetle

Analogous to *C. elegans*, this process is called “recovery” and the recovery-inducing signal is the “food signal” (Riddle et al. 1997). During recovery, the DJ release the symbiont cells into the insect’s haemocoel. The bacteria produce toxins and other metabolites (Dunphy and Webster 1988; Bowen et al. 1998), which contribute to overcome the insect’s defence mechanisms and kill the insect within approximately 2 days after nematode invasion (Simoes and Rosa 1996). *Heterorhabditis* spp are unable to kill an insect without the presence of *P. luminescens* (Han and Ehlers 2000), whereas some *Steinernema* spp also produce toxins that contribute to the pathogenicity of their symbionts (Ehlers et al. 1997). The bacteria proliferate and produce suitable conditions for nematode reproduction. Feeding on the symbiont cells, they develop into adults and produce offspring. As long as abundant nutrients are available, additional adult generations develop. When the nutrients are consumed, the offspring develop into DJ, which retain the symbiotic bacteria in the intestine (Popiel et al. 1989) and leave the insect cadaver in a search for other hosts (Fig. 1).

The symbiotic bacteria

Only a few strains of the symbiotic bacteria have been described and studied in detail. Their molecular biology has been described by Forst and Nealson (1996). Since then, the symbionts have gained considerable attention, due to commercial interest in insecticidal metabolites active on ingestion by the insect and causing symptoms in the gut similar to the *Bacillus thuringiensis* δ-endotoxin (Blackburn et al. 1998). The genes represent a possible alternative to *B. thuringiensis* toxin genes for expression in transgenic plants (Guo et al. 1999). Due to this discovery, sequencing of *P. luminescens* is now underway (Ffrench-Constant et al. 2000).

Typical for symbionts of both genera is the phenomenon of phase variation, the two extremes of which are the primary and the secondary phase (Akhurst 1980). Intermediate phases have been reported (Gerritsen and Smits 1997). The primary phase is isolated from the DJ or infected insects, whereas the secondary phase occurs either after in vitro subculturing or in vivo, when the nematodes emigrate from the cadaver (Grunder 1997). The secondary phase is not retained by the DJ of *H. bacteriophora* (Han and Ehlers 2001). Krasomil-Osterfeld (1995) induced the secondary phase by cultivating a primary form under stress conditions, for instance in media with low osmotic strength. When the bacteria were subcultured under standard conditions, they reverted to the primary phase. Prolonged subculture under stress conditions produced stable secondary phase cultures. Despite the loss of several metabolic functions by the secondary form, like production of protease, lipase, intracellular crystalline proteins, antibiotics and pigments (Boemare and Akhurst 1988), the major drawback is that secondary phase bacteria can have a significant and detrimental effect on nematode development and yields (Ehlers et al. 1990; Völgyi et al. 1998; Han and Ehlers 2001). The crystalline inclusion protein is of major importance for nematode nutrition (Bintrim and Ensign 1998). However, it is not the only essential nutritional factor provided by the primary phase (Hussein and Ehlers 2001). All measures should therefore be taken to avoid the phase variation. In general, the phase shift can be prevented by carefully reducing stress (lack of oxygen, high temperature, deviation from optimal osmotic strength of medium) during bacterial inoculum production, inoculation and the pre-culture. The mechanisms causing the phase

Table 1 Commercial use of entomopathogenic nematodes. Applications against pest insects marked *m* are currently under development for market introduction

Common name	Scientific name	Order	Culture
Fungus gnats	*Lycoriella solani*, *L. auripila*, *Lycoriella* spp	Diptera	Mushrooms
Fungus gnats	*Bradysia coprophila*, *Bradysia* spp	Diptera	Ornamentals
Cabbage root fly^m	*Delia radicum*	Diptera	Cabbage
March flies	*Bibio hortulans*	Diptera	Turf
House fly^m	*Musca domestica*	Diptera	Stables
Leafminer	*Liriomyza* spp	Diptera	Ornamentals, vegetables
Black vine weevil	*Otiorhynchus sulcatus*	Coleoptera	Strawberry, ornamentals
Strawberry weevil	*O. ovatus*	Coleoptera	Strawberry, cranberry
Hop weevil^m	*O. ligustici*	Coleoptera	Hop
Sugarbeet weevil	*Temnorhinus mendicus*	Coleoptera	Sugarbeet
Citrus root weevil	*Diaprepes abbreviatus*	Coleoptera	Citrus
White grubs	*Popillia japonica*, *Anomala* spp, *Phyllopertha horticola*, etc.	Coleoptera	Turf
Peanut white grub^m	*Maladera matrida*	Coleoptera	Sweet potatoes, peanuts
Cutworms	*Agrotis ipsilon*, etc.	Lepidoptera	Various
Banana moth	*Opogona sacchari*	Lepidoptera	Ornamentals
Ghost moth	*Hepialus* spp	Lepidoptera	Chives, ornamentals
Cockroach^m	*Periplaneta americana*, *Blatta* spp	Blattaria	Household
Mole cricket	*Scapteriscus* spp	Orthoptera	Turf
Western flower thrips^m	*Frankliniella occidentalis*	Tysanoptera	Ornamentals, vegetables
Cat flea	*Ctenocephalides felis*	Siphonaptera	House gardens

transition are yet unresolved, although genetic variation has been excluded (LeClerc and Boemare 1991; Akhurst et al. 1992; Wang and Dowds 1993).

Several interactions have been identified in the symbiosis between the nematode and the associated bacterium. During the DJ stage, the bacterium is protected from the competitive conditions in the soil and is vectored into a sterile environment. During pathogenesis, the symbionts co-operate in overcoming the insect's defence mechanisms (e.g. Götz et al. 1981). In contrast to other rhabditid nematodes, which can feed and reproduce on a variety of different bacteria, EPN have developed a close symbiotic relation with their particular symbiont species. In bacteria-free insects, steinernematids produced a limited number of offspring, but heterorhabditids cannot develop beyond the first juvenile stage (Han and Ehlers 2000). Many symbiotic mechanisms within the nematode–bacterium complex await further elucidation.

Commercial use in biocontrol

EPN have several advantages that qualify them as commercially valuable biocontrol agents. They are highly effective and often surpass the control results achieved with chemical compounds. In contrast to chemicals, which should not be displaced by water in the soil and have to decay within a few days, EPN are mobile and persistent. They recycle inside the host insect (Fig. 1), thus causing long-term, sustainable effects on the pest populations (Peters 1996). The use of EPN is safe for both the user and the environment. They have little detrimental effects on non-target insect populations and neither the nematodes nor their bacterial associates cause any detrimental effect to mammals or plants (Bathon 1996; Ehlers and Hokannen 1996). In almost all countries, EPN are exempted from registration requirements, which enables small and medium-sized enterprises to develop nematode-based plant protection products. EPN can be stored for some months, which facilitates the marketing of nematode-based products. DJ are resistant to shear forces and can thus be applied with conventional spraying equipment. As the control potential of EPN is not limited by customary agrochemicals, they can be integrated into standard chemical control practice. Today, nematodes are mainly used in environments where chemical compounds fail, i.e. in the soil, in the galleries of boring insects, or in cases where resistance to insecticides has developed (Table 1).

History of nematode in vitro mass production

An important prerequisite for the use of an antagonist in biocontrol is its mass production at low cost. In vivo propagation is too laborious and can therefore be excluded. When, for the first time in history, EPN were used to control larvae of the Japanese beetle (*Popillia japonica*) in the USA, Glaser (1931) had already tried to mass-produce *S. glaseri* in vitro. The presence of its symbiotic bacterium was not yet known and efforts to continuously produce EPN therefore failed. Early attempts to grow *Steinernema* spp in axenic culture were successful (Stoll 1952), but the yields were too low and the media, containing unsterilised raw rabbit liver, were difficult to produce and too expensive for the system to be exploited for mass production. Although Bovien (1937) first observed bacteria inside the DJ of *S. feltiae*, many years elapsed until Poinar and Thomas (1966) described the significance of the bacterium *X. nematophilus* for the reproduction of *S. carpocapsae*. This study laid the basis for in vitro production. Only the presence of the symbiotic bacterium in monoxenic cultures produces suitable conditions for nematode reproduction with high numbers of offspring. EPN were grown on Petri dishes using different agar media (House et al. 1965; Wouts 1981; Dunphy and Webster 1989). A major progress in mass production was achieved when Bedding (1981) published his results on the growth of *Steinernema* spp on a three-dimensional medium in flasks, using polyether-polyurethane sponge as an inert medium carrier. The solid-state production has several advantages. Little investment in biotechnological equipment is necessary and the risk of process failure is partitioned over several, small production units. In developing countries, this system is still superior to liquid culture technology (Bedding 1990; Ehlers et al. 2000). However, when it comes to large-scale production, the disadvantages are overwhelming. The solid state culture is labour-intensive, vulnerable to contamination and can hardly be monitored on-line. The uneven distribution of the nematodes in the medium prevents systematic sampling and thus improvement of the technique. An exploitation of the potential of EPN for plant protection required the development of liquid culture technology.

First attempts to culture EPN in liquid media were made by Stoll (1952). He used a liquid medium containing raw liver extract and incubated the cultures on a shaker. The nematodes developed and produced offspring reaching approximately 400 DJ/ml. Stoll then made several important observations: the optimum temperature was 21–26 °C, the pH of the medium increased during culturing and media prepared at a pH of 6.0–6.5 were most productive. He cultured in the dark, because cultures incubated in light usually failed. A significant step forward was achieved by Buecher and Hansen (1971). By simply bubbling sterile air through liquid media in bottles, they could show that bubbling is an acceptable means of supplying aeration and that nematodes tolerate the shear stress related to forced aeration of the cultures. This opened the door for mass production in liquid media, as bubbled cultures provide the potential for scaling-up production volumes. However, axenic cultures could not be used to produce nematodes for biocontrol purposes, due to low yields, the high cost of media components and the absence of the symbiotic bacteria in the culture, which are necessary to achieve a rapid killing activity against the target insect (Ehlers et al. 1997). It was then generally assumed that monoxenic liquid cultures would also not be viable, because Bedding (1984) mentioned that even gentle agitation of the solid cultures suppressed nematode development. How could necessary amounts of oxygen be provided for mixed cultures of nematodes and bacteria without preventing male nematodes from inseminating females and without damaging fragile adult stages through shear forces?

The answer was given in the first patent on nematode liquid culture production, filed by Pace et al. (1986). They cultured nematodes in a standard 10-l bioreactor (Braun Biostat E) and showed that shear from a flat-blade impeller, expressed as an impeller tip velocity of 1 m/s or greater, leads to the disruption of adult females. They therefore recommended shear to be less than 0.3 m/s for maximum yields. Using a kidney homogenate-yeast extract medium, they inoculated a culture of *X. nematophilus* 24 h prior to the inoculation of 2,000 DJ/ml, using the nematode *S. carpocapsae*. When the nematodes were inoculated, the temperature was reduced from 28 °C to 23 °C and the velocity of the impeller set at 180 rpm to maintain 20% oxygen saturation. After 10 days, the culture yielded 40,000 DJ/ml. In order to increase yields and reduce losses caused by shear stress, they replaced the conventional flat-blade impeller with a paddle stirrer.

The first commercial application of the liquid culture technology was made by the company Biosys (Palo Alto, Calif.). The company was incorporated in 1987 and soon started to produce liquid cultures. In 1992, large-scale production of *S. carpocapsae* began and was scaled-up to volumes of 80,000 l. Today, a few companies still produce in solid culture, for instance, Bionema (www.bionema.se) and Andermatt Biocontrol (www.biocontrol.ch). The majority of EPN products result from liquid culture and they are produced by three European companies: E-Nema GmbH (www.e-nema.de), Koppert B.V. (www.koppert.nl) and Microbio Ltd (www.microbio-group.com).

Liquid culture process technology

Liquid cultures of EPN are particularly vulnerable to contamination. The presence of any non-symbiotic microorganism will reduce nematode yields and prevent the subsequent scale-up. As a nematode process can last up to 3 weeks, maintenance of sterile conditions is a challenge for process engineers. The monoxenicity of the cultures must already be ensured at the time of inoculum production. The symbiotic bacteria can easily be isolated from nematode-infected insect larvae. Stock cultures are mixed with glycerol at 15% (v/v), and aliquots are frozen at –80 °C. Details on the determination of the symbiotic bacteria are provided by Boemare and Akhurst (1988). More laborious is the establishment of bacteria-free nematodes. Surface-sterilised DJ should not be used, because this procedure cannot exclude the presence of contaminants. The preparation of nematode inoculum is preferably done with nematode eggs obtained from gravid female stages. Detailed descriptions about the production of monoxenic nematode inoculum are provided by Lunau et al. (1993) and Han and Ehlers (1998). Monoxenic cultures can be stored on shakers at 20 rpm and 4 °C for several months until they are inoculated into the bioreactor. Strain collections of nematodes can be kept in liquid nitrogen (Popiel and Vasquez 1991).

Owing to the potential of *Xenorhabdus* and *Photorhabdus* spp to metabolise almost every kind of protein-rich medium, the selection of appropriate culture media for EPN production can largely follow economic aspects.

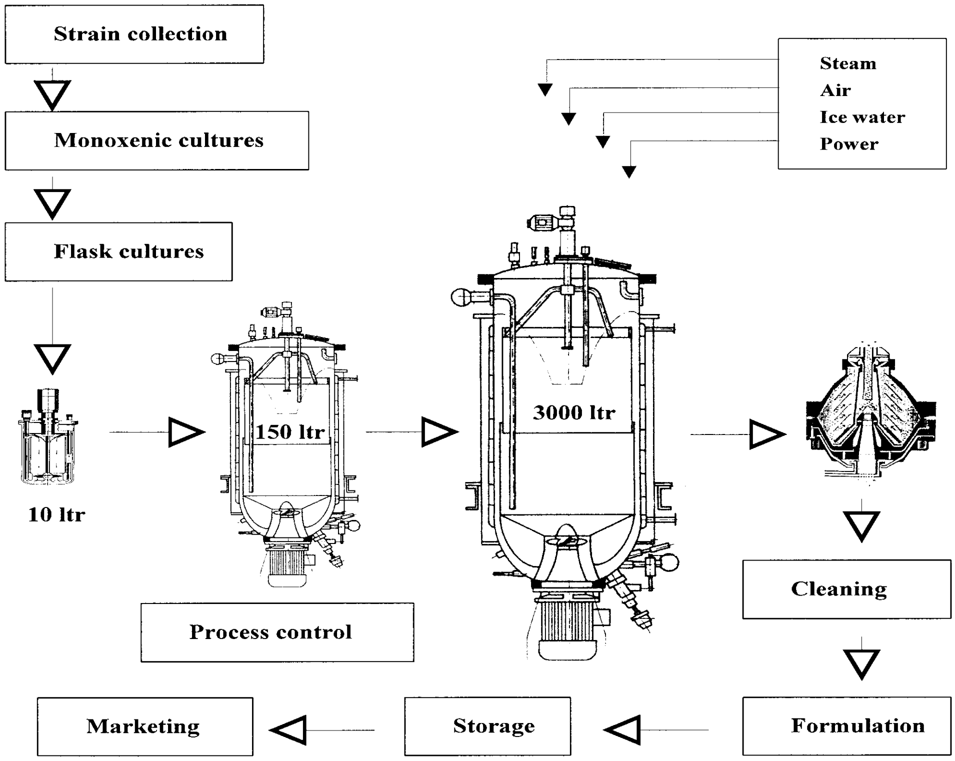

Fig. 2 Flow chart of nematode production process. After monoxenic cultures are established, they are scaled-up to a 3,000-l internal loop bioreactor. The dauer juveniles are harvested 12 days later, with a separator. The nematode paste is then cleaned by passage through centrifugal sifters and is formulated in clay

A standard medium to start with should contain a carbon source (e.g. glucose or glycerol), a variety of proteins of animal and plant origin, yeast extract and lipids of animal or plant origin (e.g. Pace et al. 1986; Friedman et al. 1989; Han et al. 1993; Surrey and Davies 1996; Ehlers et al. 1998). When composing the concentration of different compounds and minerals, the osmotic strength of the medium must not surpass 600 milliosmol/kg. Although improvements of the medium result in increasing process yields (Han et al. 1992), a systematic approach is often hindered by the variability of the nematode population development. Consequently, progress described in patents on media components (e.g. Friedman et al. 1989; Tachibana et al. 1995) should not be overestimated. For commercial production, maximum yields are less important than mean yields and process stability. Therefore the influence of media compounds on DJ recovery and nematode population development takes priority. Essential amino acid requirements have only been defined for *S. glaseri* (Jackson 1973). Nematodes have nutrititional demands for sterols, but they can metabolise the necessary sterols from a variety of steroid sources (Ritter 1988), which are provided through the addition of lipids of animal or plant origin. In general, *S. carpocapsae* requires proteins of animal origin (Yang et al. 1997) and it is unable to reproduce without the addition of lipid sources to the medium, whereas *H. bacteriophora* produces offspring in a liquid medium without the addition of lipids (Han and Ehlers 2001). Although *P. luminescens* provides or metabolises essential lipids, some lipids should always be added to increase the total DJ fat content. The lipid composition of the medium has an effect on the fatty acid composition of the bacteria and DJ (Abu Hatab et al. 1998), however, it is not known whether variable fatty acid composition influences the nematode field performance.

Equipment used in biotechnology, such as conventional bioreactors, stirred with flat-blade impellers, bubble columns, air-lift and internal loop bioreactors, have been successfully tested (Pace et al. 1986; Surrey and Davies 1996; Ehlers et al. 1998). In a direct comparison with flat-blade impeller-stirred tanks (Ehlers, unpublished) or air-lift bioreactors (Peters, unpublished), internal loop bioreactors always yielded higher DJ concentrations. Figure 2 provides an overview on the production process. Cultures are always pre-incubated for 24–36 h with the specific symbiont bacterium before DJ are inoculated. The inoculum density for the symbiotic bacterium is between 0.5% and 1% of the culture volume. A rule for the inoculation of the nematodes cannot be given, as it can vary from species to species and with media composition. An optimal number of adults/ml can be calculated, however, the adult density is defined by the percentage of DJ bound to recover (see the section on nematode population dynamics). Usually, the nematode inoculum is between 5% and 10% of the culture volume. Process parameters favouring the growth and reproduction of the nematode–bacterium complex have not yet been studied systematically and only a few results have been published. The optimum growth temperature for the symbiont of *H. indica* was investigated under continuous culture conditions (Ehlers et al. 2000) and optimum growth was recorded between 35 °C and 37 °C. Optimal culture temperature should always be defined prior to the

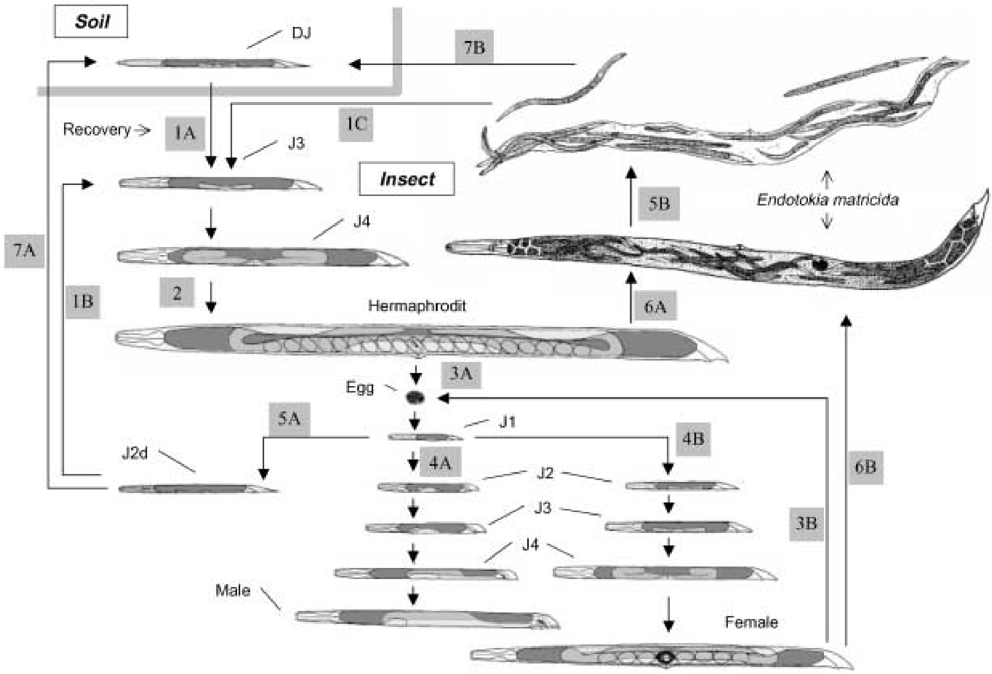

Fig. 3 Detailed life cycle of a *Heterorhabditis* sp., with alternative developmental pathways. *Numbers* indicate the critical developmental steps during the process. *1* Recovery of dauer juvenile (*DJ*) from free-living stage (*1A*), pre-dauer stage (*J2d*) originating from laid eggs (*1B*) or from *endotokia matricida* (*1C*). *2* Development of hermaphrodite. *3* Egg laying by automictic hermaphrodite (*3A*) or amphimictic female (*3B*). *4* Development to amphimictic male (*4A*) and female (*4B*). *5* DJ formation of J1 originating from eggs laid (*5A*) or from *endotokia matricida* (*5B*). *6 Endotokia matricida* of hermaphrodite (*6A*) or amphimictic female (*6B*). *7* DJ emigration of DJ originating from eggs (*7A*) or from *endotokia matricida* (*7B*). See text for further explanations

mass production of a new isolate. Any deviation surpassing the optimum can induce the formation of the secondary phase, which impedes nematode reproduction. The culture medium should be between pH 5.5 and 7.0 when the culture is started. Until nematode harvest, the pH is constantly rising; and attempts to control the pH at 7.0 always had a negative influence on nematode yields (Ehlers, unpublished). The pH appears to be well regulated by the organisms themselves. Oxygen supply must be maintained at approximately 30% saturation, also to prevent the bacteria from shifting to the secondary phase. An important parameter is the aeration rate. Strauch and Ehlers (2000) compared the yields of *H. megidis* in 10-l bioreactor cultures aerated at 0.3 vvm and 0.7 vvm and they obtained a significantly higher number of adult nematodes 8 days after DJ inoculation and a higher DJ final yield in the cultures aerated at 0.7 vvm. Increasing the aeration rate often increases foaming. The addition of silicon oil usually prevents foaming. However, it should be used carefully, because higher concentrations can be detrimental to the nematodes. Long-chain fatty acids tested to control foaming had negative effects on *H. bacteriophora* (Ehlers, unpublished). Data on final DJ yields from liquid culture have been reported by many authors (Pace et al. 1986; Bedding et al. 1993; Han 1996; Surrey and Davies 1996; Ehlers et al. 1998; Strauch and Ehlers 2000). Maximum yields of >500,000 DJ/ml were recorded by Ehlers et al. (2000) for *H. indica*. Yields showed a negative correlation with the body length of the DJ, which is first of all genetically defined and, although quite stable within a species, differs according to strain and culture conditions. However, if a species with a small DJ has the same control potential as a species with a long DJ, then the former species will always be cheaper to produce.

Nematode developmental biology

EPN mass production is linked to a great variability in yield and process stability. Major reasons for failures are the limited possibilities to manage nematode population dynamics. In order to understand the critical phases during the process, nematode developmental biology needs to be explained in more detail. Figure 3 presents the life cycle of *Heterorhabditis* spp, including alternative pathways and developmental steps, as indicated by numbers. In principal, the development is driven by the availability of food: low food concentration induces DJ formation, whereas high food concentration induces the development of additional adult generations or the recovery of the DJ. As the DJ (Fig. 3, upper left corner) is developmentally arrested, it can be stored until needed for process inoculation. Once inoculated into the culture of the symbiont, the DJ recover development (step 1A). The resulting J3 develop through the J4 to hermaphrodites (step 3), which are automictic (self-fertilizing). The final yield can already be predicted from the density and length of the hermaphrodites (Johnigk and Ehlers, unpublished results). Both the length of the hermaphrodites and the number of eggs that will be laid are positively correlated with food supply. At first, the hermaphrodites lay eggs into the surrounding medium (step 3A). Some 12 h after the J1 hatch, male phenotypes can be identified (step 4A). After another 12 h, female phenotypes are distinguishable (step 4B). In the insect or on solid media, the amphimictic adults copulate and produce another generation (egg-laying female, step 3B). In liquid media, however, the male is unable to attach itself to the female for insemination (Strauch et al. 1994). Consequently, the development ends at this point and females only contain unfertilised eggs, identified by the enlarged nucleus

(Fig. 3). Only automictic offspring can continue the life cycle in liquid media, which are a result of DJ formation (steps 5A, B). The DJ is always bound to become an automictic hermaphrodite. The decision for the development to amphimictic adults or to DJ occurs during the J1 stage. High concentrations of food induce the development of amphimictic adults (step 4), whereas low concentrations induce DJ formation (step 5A; Strauch et al. 1994). This mechanism is valid for nematodes of both genera. If the DJ have not yet emigrated from the infected insect (step 7A), the late J2d recover and continue their development to the hermaphrodite (step 1B) to produce another generation of offspring.

When egg-laying by the parental hermaphrodites ceases, the juveniles hatch inside the uterus and *endotokia matricida* (intra-uterine birth, causing maternal death) starts (step 6A). High food concentrations delay the beginning of the *endotokia matricida* and consequently enhance the number of eggs laid (Johnigk and Ehlers 1999). The length of the hermaphrodite defines the number of offspring in the uterus. The first hatched J1 immediately feed on sperm, non-fertilised eggs and oogonia, so once *endotokia matricida* has started, no further offspring can develop. In the uterus, the DJ formation (step 5B) is induced by the high nematode density and low food resources. A rapid change in food supply occurs when the juveniles have destroyed the uterus and intestinal tissues. They then have access to the body content of the adult and to cells of the symbiotic bacteria, which they retain in their intestine. Food provided by the body content of the hermaphrodite is well tuned to feed the defined number of offspring in the uterus. The resulting DJ accumulate a maximum of fat reserves and are of excellent quality (Johnigk and Ehlers 1999). Only in insects and solid cultures is *endotokia matricida* also observed in amphimictic females (step 6B). Emigrating DJ either result from DJ that have developed from laid eggs (step 7A) or from *endotokia matricida* (step 7B). *Steinernema* spp have a similar life cycle, except that only amphimictic adults are produced. The typical copulation behaviour of *Steinernema* males, which coil themselves around the female, is not impeded in liquid culture.

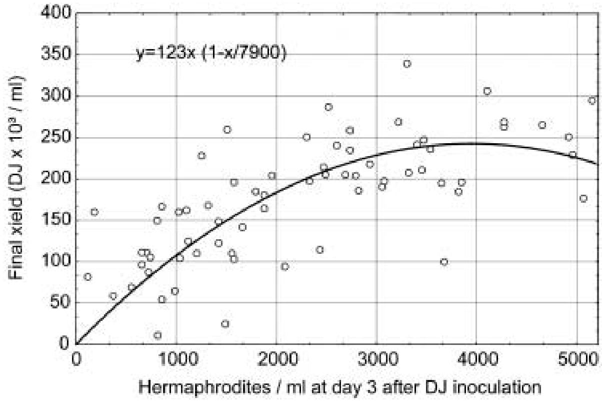

Fig. 4 Final DJ yields/ml in relation to the density of hermaphrodites on day 3 after nematode inoculation. Data are from commercial production of *H. bacteriophora* in internal loop bioreactors. The graph fitted to the data represents a model assuming a density-dependent decrease in hermaphrodite fecundity (Newton approximation, using least-squares as loss function)

Management of nematode population dynamics in liquid culture

It can be expected that a certain medium has a fixed potential for a defined nematode yield. However, yields in the same medium can vary considerably (Ehlers et al. 1998; Strauch and Ehlers 2000). The reason why the population dynamics are so important becomes apparent when data obtained from the commercial production are analysed. In Fig. 4, the relationship between hermaphrodite density and DJ yields is presented for *H. bacteriophora* (Peters and Ehlers, unpublished results). The same medium was used for all processes. Maximum yields were obtained with approximately 4,000 hermaphrodites/ml counted on day 3 after nematode inoculation. Consequently, an inoculation of >4,000 DJ/ml should result in maximum yields. This hermaphrodite density, however, cannot be obtained by defining the DJ inoculation density, because DJ recovery is highly variable in liquid culture. Whereas almost 100% of the DJ recover within 1 day after having entered the haemocoel of an insect, liquid media lack any kind of food signal that could trigger recovery. Fortunately, the symbiotic bacteria produce such food signals and they therefore enable the production of EPN in vitro, through pre-culturing the symbiotic bacteria. However, the bacterial food signals cause 18–90% of the DJ to recover within a period of several days (Strauch and Ehlers 1998). The variable hermaphrodite density recorded 3 days after DJ inoculation (Fig. 4) is a result of variable recovery. The main reason for unstable DJ yields is the unpredictable, unsynchronised and low DJ recovery from in vitro cultures. It prevents the population management required to maximise yields and to shorten the process time and makes additional scale-up steps necessary.

Low recovery results in a low hermaphrodite density. At a density of <1,000/ml (Fig. 4), the abundance of food causes the hermaphrodites to lay many eggs, from which the majority develop into amphimictic adults, instead of DJ. Although it prolongs the process time, this is acceptable when culturing steinernematids, as the amphimictic adults can copulate in liquid culture and produce a F2 offspring generation. It usually results in process failure in heterorhabditid cultures, as the F1 amphimictic adults cannot produce offspring. By the time reproductive F1 generation hermaphrodites have developed from J2D (step 5A) or from *endotokia matricida* (step 5B), amphimictic adults have consumed much of the bacterial culture. Offspring production of the F1 hermaphrodites is low therefore and those F1 that remained in the DJ stage (steps 7A, B) are of low quality, as they have already consumed part of their fat reserves at the moment of harvest. The reason why, even at a density of <1,000 hermaphrodites/ml, yields can surpass 150,000 DJ/ml (Fig. 4) is due to the potential of the hermaphrodites to respond with increasing body length and high number of offspring. This is only observed in cases of synchronous DJ recovery. With increasing numbers of hermaphrodites (>2,000/ml), their feeding activity reduces the bacterial concentration. Less offspring develop into amphimictic adults, but many develop into DJs and remain in this stage. The yield increases, until a point is reached where the hermaphrodites hardly lay any eggs and almost all offspring originate from *endotokia matricida*. This composition of the nematode population results in high yields of high quality DJ within a minimum of process time. The model (Fig. 4) indicates that, surpassing 4,000/ml, the yields then decline. Competition for food reduces the number of DJ/hermaphrodite. When the number of hermaphrodites is too high, the resources go into the basic maintenance of the adult instead of DJ production and the yields decline. Observations from flask cultures have shown that the body length of the hermaphrodites decreases, although the point of decreasing yields might be reached beyond a hermaphrodite density of 4,000/ml (Johnigk and Ehlers, unpublished results).

Recovery

As already mentioned, the key for the improvement of the process technology of *Heterorhabditis* spp is a synchronised, reproducible and high DJ recovery, in order to reach an optimum number of parental hermaphrodites. To increase DJ recovery, several process parameters were investigated (Table 2). Recovery can even be influenced during the bacterial pre-culture. The higher the bacterial density, the higher the food signal concentration. Nematodes should therefore not be inoculated too early, as the food signal concentration increases until the stationary growth phase is reached (Strauch and Ehlers 1998). The moment when the conditions become favourable coincide with a significant drop in the respiration

Table 2 Parameters influencing dauer juvenile (DJ) recovery (Strauch and Ehlers 1998; Jessen et al. 2000; Ecke, Johnigk, Böttcher, Ehlers unpublished results)

Process parameter/culture condition	Effect on DJ recovery
Food signal insect haemolymph	++
Food signal symbiotic bacterium	+
Compounds in artificial media	–
High bacterial density	+
Symbiont culture in stationary phase	+
pH within 6.5–9.0	+
pH <6.5	–
Increasing CO_2 concentration	+
DJ originate from laid eggs	+
DJ originate from *endotokia matricida*	–
Age of DJ	variable
DJ fat reserves	variable

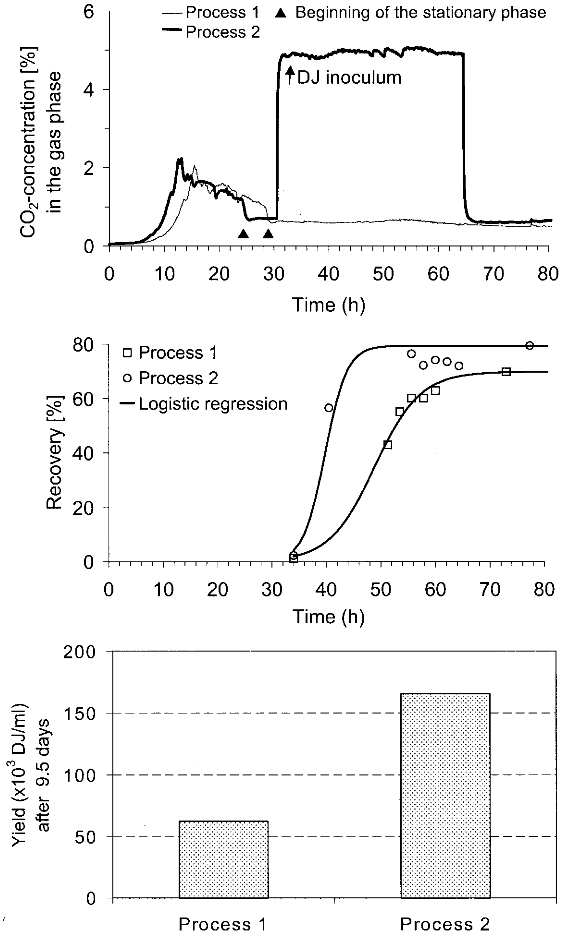

Fig. 5 Influence of CO_2 concentration on the DJ recovery (%) and final DJ yields of two parallel bioreactor runs in 5-l internal loop bioreactors. Process 1 was run under standard conditions and Process 2 received a high concentration of CO_2, to reach 5% in the exhaust gas. DJ inoculation was after the symbiotic bacteria had entered the stationary growth phase

coefficient and a drop in the pH (Ecke, Johnigk and Ehlers, unpublished results). Fed-batch cultivation, adding glucose at the end of the exponential growth, is a possible measure to increase bacterial density (Jeffke et al. 2000) and to enhance food signal production. Glucose fed-batch can thus be used to increase DJ recovery (unpublished results).

When DJ invade an insect, they enter into an environment of increased CO_2 concentration. An influence of CO_2 on DJ recovery was therefore investigated in recovery bioassays. Jessen et al. (2000) found that increasing the CO_2 concentration in the medium enhanced DJ recovery. The influence of decreasing pH caused by the CO_2 concentration was excluded. A pH below 6.5 significantly reduced the DJ recovery. The positive effect of CO_2 could be confirmed by comparing two parallel bioreactor runs, one at standard conditions and one with a CO_2 concentration at 5%. Cultures were inoculated with DJ of the same origin at 12,000/ml. The artificial increase of the CO_2 resulted in a higher percentage of DJ recovery and caused the DJ to recover earlier. The yields were more than doubled (Fig. 5).

When the response of different DJ batches is compared at standard conditions it becomes obvious that a major source for variability are the DJ themselves (Strauch and Ehlers 1998; Jessen et al. 2000). The response to the food signal differs considerably from batch to batch. This difference may be due to the variable fat reserves of the DJ. The lower the energy reserves, the higher would be the predisposition of the DJ to recover. Several experiments that tested the influence of DJ ageing (loss of fat reserves) did not support this hypothesis. A non-significant increase is usually recorded after storing DJ inoculum for 1 week, although the increase in DJ recovery is often frustrated by increasing DJ mortality during storage. The only significant difference was recorded for DJ originating from *endotokia matricida* or from laid eggs. The latter had a significantly increased predisposition to recover (Ehlers, unpublished results).

Conclusions

Major problems related to EPN liquid culture mass production remain unsolved. Physiological parameters that cause one DJ to respond to the bacterial food signal and another to remain in the DJ stage remain unknown. Another source for process instability results from the phase transition of the bacteria. Both fields deserve further investigation, in order to enhance process stability and increase yields. Comparing the nematode process with the cultivation of *Escherichia coli* or other microorganisms, very little is known about nematode cultivation. The close relation of EPN to the model nematode *C. elegans* and the sequencing project on *P. luminescens* will hopefully yield some background information about the metabolism of the nematode–bacterium complex which will be valuable for improving process technology. Additional research on the symbiosis and its genetic background should identify the essential growth factors provided by the bacterium and elucidate the function of the phase transition.

At this moment, EPN are taking the step towards outdoor environments (turf grass and strawberries). In vegetable and fruit, many pests exist that can be controlled by EPN. However, these potential markets will only demand nematode products when these are available at a lower price. Although the price has been cut by half following the introduction of liquid culture technology, it is still considerably too high to permit any application on low-value crops. The continuous scale-up of bioreactor volumes will bring further reductions in the production costs. However, this development must be accompanied by further progress in improving process stability and downstream processing, in measures to extend EPN shelf life and in improving transport logistics. If this can be achieved, EPNs will further substitute insecticides and contribute to the stabilisation of agriculture environments and crop yields.

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