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Bioreactor Cultivation of the Nematode *Caenorhabditis elegans:* Large Scale Production of Biologically Active Drug Receptors for Pharmaceutical Research

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Introduction

There is growing interest within the biotechnology community in the study of nematodes, particularly the free-living nematode *Caenorhabditis elegans.* The fascination of scientists with *C. elegans* for both basic and applied research is due to the convergence of a number of current trends in modern biology. The selection of the free-living nematode *C. elegans* as a model organism for elucidating the genetics and developmental biology of multicellular organisms has led to an explosion in biochemical knowledge about *C. elegans* (Brenner, 1974). The hallmarks of the concerted research efforts on *C. elegans* over the last three decades are summarised in *Table 1*.

As a result of these advances in knowledge, *C. elegans* has become one of a select group of model organisms including bacteriophages, *Escherichia coli*, *Drosophila melanogaster* (fruit flies) and *Arabidopsis thaliana* (Thala weed, a plant species) whose detailed biochemical analysis established the cornerstones of molecular biology. The choice of *C. elegans* as an investigative model in modern biology is partly due to considerations of convenience, including ease of growth and handling to enable controlled laboratory experimentation, and partly because of the simplicity of its biological organisation. One outcome of these concerted studies is the proliferation of laboratory techniques for culture and experimentation with these organisms.

Besides the free-living nematodes, parasitic nematodes are responsible for numerous diseases, in humans as well as in domestic animals and plants. Damage to agricultural crops due to parasitic nematodes is estimated to run at over $5 billion dollars annually in the USA alone (Wood, 1988). *Table 2* lists some major parasitic diseases caused by nematodes in animals and man.

The devastating impact of parasitic nematodes on public health and agriculture has led to extensive applied research to identify vulnerable, nematode-specific targets for drug intervention and pharmaceutical development. This constitutes part of the

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Table 1. Highlights of research on *C. elegans*

- Estimation of size of *C. elegans* genome at 100 million base-pairs comprising 2000 genes distributed on six chromosomes
- Description of the neural network (wiring diagram) of the entire nervous system of *C. elegans*, consisting of 302 neurons (White *et al.*, 1986)
- Tracing the complete cell lineage of *C. elegans* identifying the origin of all 959 cells (Horotivz, 1988)
- Physical map of the *C. elegans* genome (Coulson *et al.*, 1986)
- Sequencing of the *C. elegans* genome (Coulson., 1988; Watson, 1990, Waterson *et al.*, 1993)

Table 2. Nematode parasitic diseases in man and animals (Markell and Voge, 1976)

Parasitic diseases	Causative nematode
Ascariasis (intestinal worm)	*Ascaris lumbricoides*
Hookworm	*Ancylostoma duodenale/Necator americanus*
Strongyloidiasis	*Strongyloides stercoralis*
Onchocerciarsis (river blindness)	*Onchocerca volvulus*
Filariasis	*Wuchereria bancrofti*
Guineaworm	*Dracunculus medinensis*
Trichinosis (from uncooked pork)	*Trichinella spiralis*

motivation for the *C. elegans* research effort at the Merck Research Laboratories which led to development of large scale cultures of nematodes for isolating receptors for the potent anti-parasitic drug, ivermectin. Ivermectin is a chemical derivative of the natural product avermectin produced as a secondary metabolite by *Streptomyces avermitilis* (Fisher and Mrozik, 1989).

Besides the interest in basic and applied research, nematodes are also attracting interest as commercially useful products such as biopesticides for agricultural use and biological agents for monitoring toxic compounds in the environment. These trends create further challenges for large scale cultivation of nematodes to meet the growing requirements of these applications.

Thus far, generic fermentation technologies designed for bioreactor cultivation of micro-organisms have proved highly adaptable to new challenges and opportunities evolving through modern biotechnology. Thus large-scale tissue culture technologies for mammalian, insect and plant cells have been successfully established, based on principles and equipment utilised for microbial cultures with relatively minor modifications.

The distinguishing feature of nematodes from the cell types hitherto cultivated is that they constitute whole organisms comprising multicellular, differentiated cells. A further complication in the culture of nematodes is the mode of reproduction through a complex developmental life cycle consisting of eggs, larvae and adult stages. In the case of *C. elegans* the self-fertilizing mode of reproduction is a critical enabling factor in adaptation to bioreactor cultivation. In principle, the peculiar biological characteristics of organisms of interest need to be carefully considered in efforts to develop bioreactor cultivation techniques,

Life-cycle of *C. elegans*

C. elegans is a free-living nematode dwelling naturally in the soil. The adult worms grow to about 1 mm in length and occur predominantly as self-fertilizing herma-

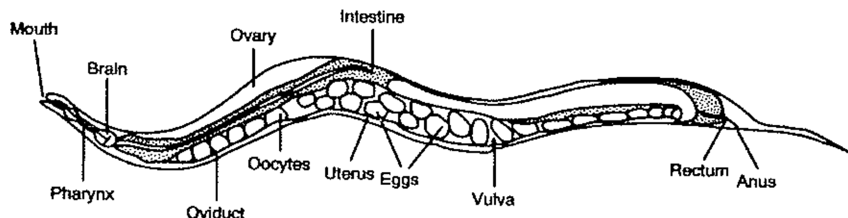

Figure 1. Structural diagram of adult hemaphrodite *C. elegans.* (Adapted from original drawing by Sulton and Horotivz, 1977.)

phrodites with an extremely low frequency of sexual male forms. The hermaphrodites consist of 959 somatic cells whilst the males consist of 1031 cells (Wood, 1988). *Figure 1* shows a diagram of the structure of an adult hermaphrodite worm depicting internal organs for feeding, digestion, excretion and reproduction. The various organ structures are clearly visible under the microscope through the transparent body of the adult *C. elegans*.

During a transient period leading to maturation, hermaphrodites produce a limited number of sperm (numbering about 150) which are stored for later use. Female reproductive organs develop as the hermaphrodites undergo a transition to produce oocytes for the rest of their reproductive life cycle, which are self-fertilized by the stored sperm. Occasionally cross-fertilization occurs when hermaphrodites come into contact with the males. However, mating is rare since males constitute less than 0.5 % of the population of *C. elegans*.

The hermaphrodites can lay up to 300 eggs during their reproductive phase. The eggs hatch within 14 h of fertilization into larvae or juvenile worms which develop and moult through four larval stages to mature into adults. *Figure 2* illustrates the life cycle of *C. elegans* marking the evolution through L1, L2, L3, L4 larval stages into the adult worm within a 3-day period. The adults normally survive for another 17 days before senescence. Under adverse conditions such as inadequate food supply, development of the L2 larva passes into the dormant dauer phase. The dauer phase can survive under adverse conditions up to 3 months before moulting resumes if favourable conditions are restored (Riddle, 1988).

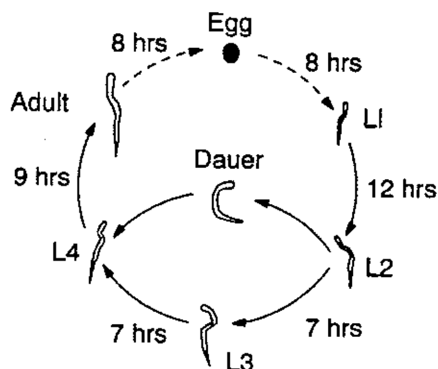

Figure 2. Life-cycle of *C. elegans* depicting development through egg, larva and adult, as well as dauer phases of growth (Riddle, 1988).

GROWTH AND NUTRITIONAL CHARACTERISTICS

The growth characteristics of *C. elegans* in culture are summarised in *Table 3* (Croll and Matthews, 1977). The free-living nematodes thrive in nature as microbivores and can be cultured on simple bacteria medium (monoxenic cultures) as well as on nutrient media supplemented with essential nutritional factors (axenic cultures). A typical complex media composition to support axenic culture of *C. elegans* consists of soy peptone, 40 g/l; yeast extract, 10 g/l; liver extract, 100 g/l. The latter component provides heme and dietary sterol which were found to be critical nutritional requirements for nematodes (Rothstein and Coppens, 1978). The heme nutritional requirement can be met by addition of hemoglobin (500 mg/l) or hemin chloride (200 mg/l).

Table 3. Growth Characteristics of *C. elegans*

Generation time (18–22°C)	4-5 days
Estimated longevity (18-22°C, growth on bacteria)	10–12 days
Longevity in axenic culture (18–22°C)	34 days
Peak population densities	200,000 worm counts/ml

Chemically defined media formulations have been developed to support axenic cultures of nematodes. The most frequently used formulation is *C. briggsae* maintenance medium (CbMM), which consists of essential and non-essential amino acids, vitamins and trace elements, supplemented by a source of sterols (Platzer, 1977). The basal salt composition of CbMM is: $CaCl_2.2H_2O$, 220 mg/l; $CuCl_2.2H_2O$, 6mg/l; $MnCl_2.4H_2O$, 22 mg/l; $ZnCl_2$ 10 mg/l; KH_2PO_4, 1125 mg/l, K_3 citrate.H_2O, 486 mg/l; $Fe(NH_4)_2(SO_4)_2$. $6H_2O$, 58.8 mg/l; MgH.citrate.$5H_2O$, 643 mg/l, cytochrome c, 50 mg/l, β-sitosterol, 50 mg/l and Tween 80 20 g/l (Lu *et al.*, 1983). Nutritional studies indicate the preferred carbon sources for growth of *C. elegans* in defined media include glucose, glycogen and trehalose (Lu and Goetsch, 1993).

In general, growth and reproduction rates of nematodes are much slower in axenic cultures (generation time, 7–8 days) than in monoxenic cultures though longevity increases under axenic conditions.

Monoxenic cultivation of nematodes

Free-living nematodes survive in nature as microbivores by ingesting bacteria from the soil. Monoxenic cultures attempt to mimic this mode of growth under controlled conditions by cultivation of nematodes on specific species of bacterial cultures. Selection of the type of bacterial species to support growth of a particular nematode appears critical to the success of this mode of cultivation. Studies reported by Wilson *et al.* (1995) indicate that there are significant variations in yield of nematodes grown on different bacteria species due to differences in bacterial attraction, mode of ingestion, digestibility and release of toxic or inhibitory substances. Thus compatibility of the nematode and the bacteria is critical in establishing monoxenic cultures; generally the natural bacterial symbiont of the nematode is the preferred species.

Monoxenic cultivation of nematodes can be achieved by simultaneous growth of the bacteria and the nematodes together in the same nutrient media. Alternatively a two-step procedure is frequently employed, involving initial growth of the bacteria, which are then harvested and resuspended in the nematode growth medium. Propo-

nents of the single-step process (Buecher and Popiel, 1989) claim advantages of convenience through combined growth of bacteria and nematode in a common nutrient medium and bioreactor. Typically a single-step nutrient medium consists of 3% tryptic soy broth, 0.5% yeast extract supplemented with cholesterol, 40 microgram/l. Normally bacterial growth is allowed to reach log phase before introduction of the nematode into the culture to ensure a stable food supply to the latter.

The single-step concurrent growth of nematodes and bacteria provides an interesting case study of population dynamics in a culture system involving predator-prey interaction between the two species. Theoretically, sustenance of a stable culture system will depend on factors such as the growth rate differential between the two species, preferred growth temperatures, as well as sensitivity to release of inhibitory waste products between the two species (Pirt, 1975).

We employed the two-step mode of cultivation in our studies with *C. elegans* (Gbewonyo *et al.*, 1994). *E. coli* strain OP50-1 (a leaky uracil auxotroph, which is resistant to streptomycin and phage 80) was mass produced in 800-l fermentations to generate bacterial cell paste. The bacterial growth medium consists of bacto-tryptone, 16 g/l; yeast extract, 10g/l; $MgSO_4 . 7H_2O$, 0.6 g/l; NaCl, 5 g/l; Ucon defoamer, 1 ml/l. Approximately 6 kg wet cell paste was harvested from each fermentation batch, centrifuged and stored at –20°C for subsequent nematode cultivation.

The procedure used for cultivation of *C. elegans* in bioreactors is described in the flowchart in *Figure 3*. The operational details have been described previously (Gbewonyo *et al.*, 1994) The seed cultures were prepared from wild type strain of *C. elegans* N2, maintained on agar plates. Two seed stages grown in shake flasks were required to generate enough nematodes to inoculate the bioreactor. The nutrient medium used for cultivation of both the seed and production stage cultures is the S medium containing a suspension of *E. coli* cells. The S medium composition is shown in *Table 4*.

Table 4. S Medium Composition (Sulston and Hodgkin, 1988)

Potassium citrate	3.1 g/l
KH_2PO_4	5.5 g/l
K_2HPO_4	1.8 g/l
NaCl	5.8 g/l
$CaCl_2$	0.3 g/l
$MgSO_4$	0.4 g/l
cholesterol	5 mg/l
disodium EDTA	19 mg/l
$FeSO_4.7H_2O$	7 mg/l
$MnCl_2.4H_2O$	2 mg/l
$ZnSO_4.7H_2O$	3 mg/l
$CuSO_4.7H_2O$	0.3 mg/l
Ucon defoamer	1 ml/l
Streptomycin	50 mg/l (added to prevent adventitious contaminants)
Nystatin	10 mg/l
E. coli cell paste	20 g/l

Bioreactor cultivation was conducted in a 280-l stirred tank with a working volume of 150 l, using the S medium containing 20 g/l suspension of *E. coli* paste. It is critical to disperse the *E. coli* cell paste with high agitation to give a fine suspension of

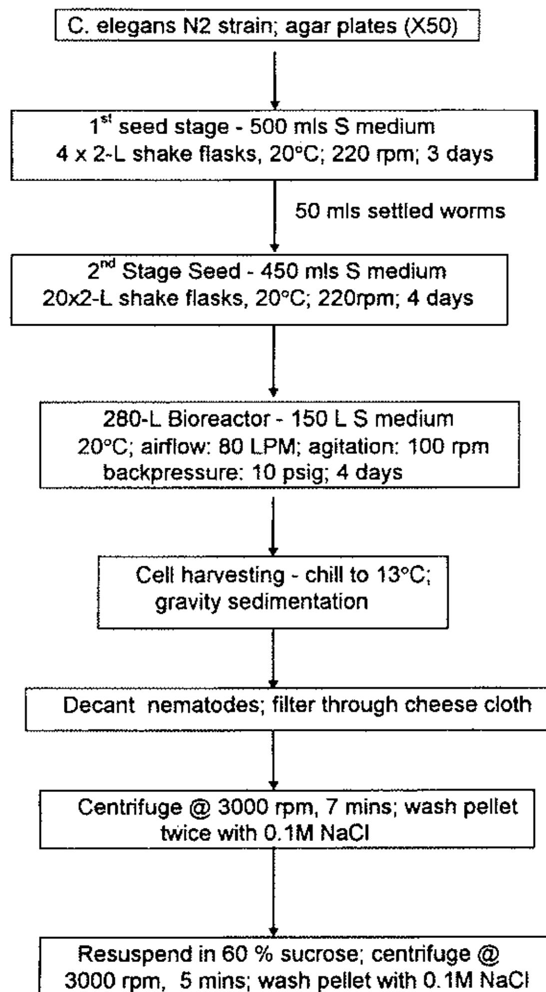

Figure 3. Flowsheet showing procedure for cultivation and harvesting of *C. elegans* in 150-l bioreactor.

discrete, single cells to facilitate ingestion by the nematodes. This is done before inoculation so that agitation was kept at a reasonably low speed of 100 rpm after addition of the seed culture in order to avoid shear damage to the nematodes. This agitation rate was found to be sufficient to keep dissolved oxygen levels above 20% saturation during cultivation.

The growth profile of the culture shown in *Figure 4* indicates that the nematode population increased significantly over a period of four days reaching above 30,000 worms per ml as measured by microscopic counts. Consumption of the *E.coli* cells is indicated by the decrease in turbidity of the culture supernatant from 8 to 3 optical density units. Time profiles of culture pH and rates of respiration are shown in *Figure 5*. The pH showed a tendency to rise and was kept between pH 6 and pH 7 by addition of phosphoric acid to the bioreactor. The rates of oxygen uptake and carbon dioxide evolution reached a peak of 4 mmole/l/h during the first day of cultivation in the

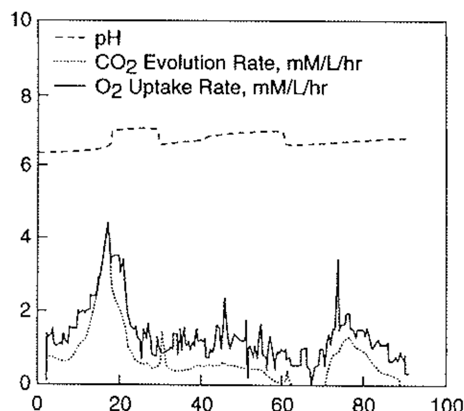

Figure 4. Growth profile of *C. elegans* population in bioreactor.

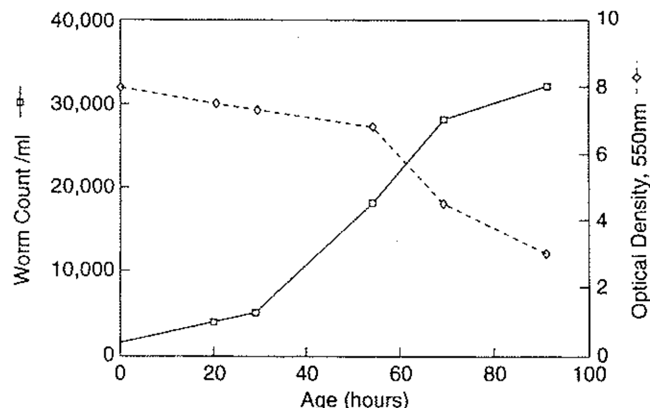

Figure 5. Time profiles of pH and respiratory rates during cultivation of *C. elegans* in 150-l bioreactor.

bioreactor. This is considerably lower than typical respiratory rates observed with microbial cultures. The low oxygen demand for nematode cultivation enabled us to keep adequate dissolved oxygen concentration with relatively mild agitation and airflow rates. The development of the nematodes during the growth cycle was followed by microphotography as shown in *Figure 6*. Within 24 h of inoculation, eggs were observed in the abdominal cavity of the adult nematodes. By the second day larval stage nematodes were released into the culture which then developed into full-size adults ready for harvest by the fourth day. The nematodes were harvested from the culture by a series of gravity sedimentation procedures as previously described (Gbewonyo *et al.*, 1994). The viable nematode fraction was recovered by sucrose gradient centrifugation yielding about 500 g wet weight of nematodes from each batch of 150-l culture.

Isolation of membrane-bound receptors (see *Figure 7*)

In order to isolate membrane-bound protein receptors for avermectin-binding studies,

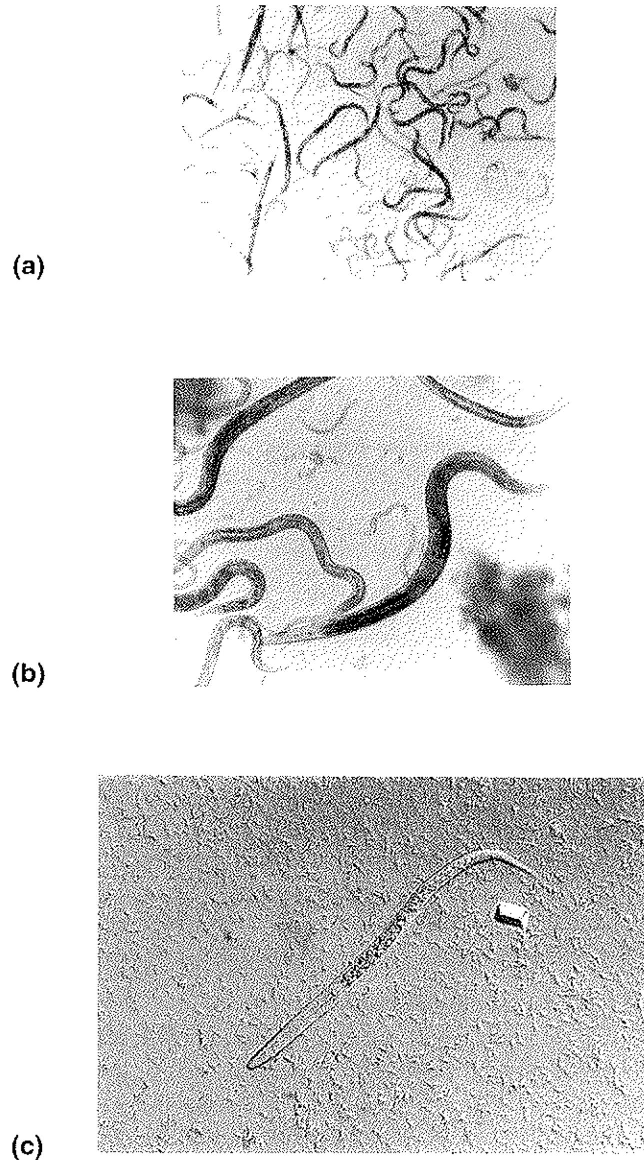

Figure 6a, b, c. Microphotographs of *C. elegans* culture samples from bioreactor showing outlines of internal organs. (**a**) 40 × magnification, (**b**) 100 × magnification, (**c**) 100 × magnification.

nematodes were resuspended in HEPES buffer containing a cocktail of protease inhibitors and homogenised through a high pressure Manton-Gaulin homogenizer. The homogenate was centrifuged twice at low speed (1,000**g** for 10 min) to discard cell debris and the supernatant was centrifuged at high speed (28,000**g** for 30 min). The pellet fraction was resuspended and dialysed overnight against a large volume of 50mM HEPES buffer containing protease inhibitors to obtain the nematode membrane concentrate.

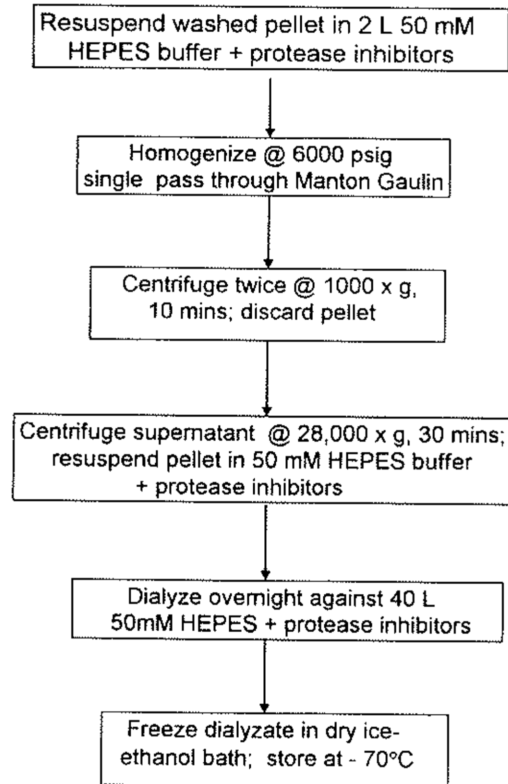

Figure 7. Flowsheet showing procedure for membrane tissue preparation from *C. elegans* biomass.

Biochemical characterisation of membrane-bound receptors.

Membrane preparations of *C. elegans* have been shown to exhibit specific avermectin binding sites (Schaeffer and Haines, 1989). These researchers demonstrated equilibrium binding of the membrane fractions with ^{3}H-labelled avermectin. The binding affinities of avermectin analogues were further shown to correlate with biological potency as measured by *in vivo* effects on *C. elegans* motility. Avermectin has been shown to increase permeability of cell membranes to chloride ions in nematodes and insects.

Non-ionic detergents were used to solubilise avermectin receptor activity from crude membrane preparations. The solubilised fractions showing high affinity for avermectin were characterised as high molecular weight complexes of polypeptides with low (acidic) isoelectric point (Cully and Paress, 1991). In order to further purify and characterise the avermectin receptors, high affinity ligand probes have been chemically synthesised (Meinke *et al.*, 1992). A radio-labelled ^{125}I-azido-avermectin derivative was used to bind selectively to receptors in detergent-solubilised membrane preparations by photo-activated cross-linking reaction. The covalently tagged protein fractions showing high avermectin binding were identified by gel electrophoresis and autoradiography to have molecular weights of 8, 47 and 53 kDa (Rohrer *et al.*, 1992).

Subsequent cloning of the *C. elegans* avermectin-sensitive, glutamate-gated, chloride channels was accomplished by expressing *C. elegans* RNA pools in *Xenopus* ooyctes and screening for avermectin-responsive chloride channels (Arena *et al.*, 1991, 1992; Cully *et al.*, 1994). These experiments with *C. elegans* tissues provide insights into the mechanism of action of the avermectins and their receptor binding sites. Many of these biochemical studies would not have been possible without techniques for large-scale cultivation of nematodes to generate sufficient cellular tissues for isolation of very rare biomolecules.

Whole cell approaches to drug/toxicity screening

Besides the use of isolated biomolecules, the applications of nematode cultures as 'whole cells' in drug screening, toxicological testing, and environmental monitoring have been reported by various researchers.

An anthelmintic bioassay screen using *C. elegans* suspensions incubated in the presence of various test compounds and extracts was described by Simpkin and Coles (1981). Detection of compound bioactivity was scored simply by microscopic examination of plates for viability and growth of *C.elegans* cultures after a suitable period of incubation.

Candido and Jones (1996) recently reported the development of transgenic strains of *C. elegans* for use as biosensors for toxicological and environmental monitoring. *C. elegans* was transformed with genetic constructs for stress protein (*hsp*) genes fused to lacZ reporter. The *hsp* gene was induced in response to adverse environmental conditions such that the reporter gene expresses β-galactosidase which is chromogenically detected by ONPG or X-gal. This bioassay system has been used successfully to detect toxic effects of heavy metals such as mercury and cadmium in environmental samples.

Scale-up challenges for mass production of nematodes

Recent technological progress in large-scale cultivation of nematodes has been largely driven by increasing demands for nematode products for use as biological control agents for insect pests in agriculture. Nematode species belonging to the families *Steinermatids* and *Heterorhabditis* in association with their symbiont bacteria, *Xenorhabdus,* have been found to be highly pathogenic to certain target insects including weevils, termites and beetles (Klein,1990). Consequently these nematode species have recently been developed and promoted as environmentally-friendly pesticides by biotechnology companies such as Biosys, (California, USA) and Ecogen, (New Jersey, USA). The major challenge faced in the commercialisation of nematode products is the development of efficient techniques for mass cultivation of the nematodes in bioreactors.

The major constraint to cultivation of nematodes in bioreactors is sensitivity of the organisms to shear stresses generated by the impellers in agitated vessels. In order to avoid shear damage to nematodes in mechanically stirred tanks, Pace *et al.*, (1986) determined that tip speed during nematode cultivation must be kept below 1 m/s. Employing a single-step process in a 10-l bioreactor, they reported up to 40,000 nematodes per ml of *Steinernema feltiae* grown in the presence of *Xenorhabdus*

bacteria. The bacteria were inoculated 24 h prior to inoculation of the nematodes, after which agitation was kept at 180 rpm with flat-blade turbine impellers. Retrofitting the bioreactor with low shear paddle impellers improved the yield to 70,000 nematodes per ml. They also found that changing the bioreactor configuration to aerated tanks in which mechanical agitators were replaced with downward pointing air sparger increased yields even further to 90,000 nematodes per ml.

The effects of shear on yield of the nematode culture was corroborated by Freidman (1990) in studies of the influence of agitation speed on nematode yields in a 20-l bioreactor as summarised in *Table 5*. At low agitation speeds (50, 125 rpm) the culture was apparently deprived of dissolved oxygen, however when agitation was kept high (500 rpm), a prolonged lag phase ensued and yield was limited presumably by shear effects. Higher yields resulted as the agitation was varied during cultivation in response to the rise and fall in oxygen demand of the culture. These researchers reported a peak oxygen demand of 20 mmoles/l/h which is attributed to the higher oxygen demand for bacterial growth. This is contrary to our experience with separate cultivation of the bacteria and nematode in the two-step process which showed a five-fold less oxygen demand for *C. elegans* cultivation. It must be noted that the conflict between oxygen demand for bacterial growth and shear sensitivity of the nematodes presents a serious drawback for the one-step cultivation process.

Table 5. Influence of Agitation Speed on *Steinernema* Yield (Friedman, 1990)

Agitation speed (RPM)	Yield (nematodes per ml)
50	30,000
125	70,000
500	70,000 (extended lag phase)
Variable speed (250–500)	110,000

Notwithstanding this limitation, workers at Biosys have reported scale-up of *Steinernema* cultivation in bioreactors up to 75,000l (Erickson, 1992). It is clear that cultivation techniques for nematodes are still in their infancy and additional studies are needed in order to cope with increasing demands for nematodes, both as research tools and as end-products for commercial applications.

More studies are needed on the physiology and nutrient requirements for nematode cultivation; special conditions favorable for the different stages of nematode life-cycle need to be defined. Further analytical models will be useful to describe the dynamics of co-cultivation of bacteria and nematodes, which will help in choosing an optimal operating strategy (i.e., one-step vs. two-step process). Studies are also needed to characterise shear sensitivity of nematodes in bioreactors as well as improve techniques for harvesting and isolation of biomolecules from nematode cultures.

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