Large Scale Cultivation of a Free-Living Nematode (*Caenorhabditis elegans*)

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Summary. A method is presented for the large scale cultivation of the free-living nematode *Caenorhabditis elegans*, using continuous aeration and agitation in glass ware (stirrer flasks) developed for the continuous culture of suspended cells. With this technique, populations up to 10^9 nematodes may be obtained in a 10 l culture in less than 6 weeks with an inoculum of some 50 worms. Costs can be reduced by using an inexpensive yeast extract, available from the food industry.

Free-living nematodes have been deemed suitable as model organisms for the study of behavioural genetics and experimental gerontology[1,2]. These organisms can be grown readily on a layer of bacteria or, under aseptic conditions, in shallow liquid media[3]. Neither of these methods, however, is suitable for the production of the large quantities of biological material that are often required for biochemical analysis. In addition, the media in current use for axenic cultivation are rather expensive and need supplementation with proteinaceous supplements that are time-consuming to prepare. The present paper reports on large scale cultivation of the free-living nematode, *Caenorhabditis elegans*, in inexpensive medium using glassware developed for the continuous culture of suspended cells.

Adequate medium for routine culture of free-living nematodes currently consists of 3% yeast extract, 3% soy-peptone and 10% heated liver extract (HLE)[4] as growth promoting supplement. In this medium HLE serves mainly as a suitable source of haem (or haemin) and may be substituted by pure acid-precipitated haemin[5]. Actually HLE was substituted with 50 µg/ml acid-precipitated haemin chloride, 1% dextrose and 1% bacto-casitone (media 4, 5, 6 and 7 in Table I). Dextrose substitutes for the carbohydrate content of HLE. Bacto-casitone was included because this nutrient has a stimulatory effect on growth of the closely related species *Caenorhabditis briggsae*[6].

It is obvious from Table I that nematode growth in media with acid-precipitated haemin is not as fast and profuse as in media supplemented with growth factor prepared from tissue extracts. Substitution of growth factor with pure haemoglobin following ROTHSTEIN[7,8], however, seems to be applicable to large scale cultivation as it is not expensive and nematode growth is almost as fast and profuse as with HLE.

Efforts were also made to substitute for Difco yeast extract and oxoid soy-peptone that are rather expensive when required in large amounts. A very inexpensive yeast extract was obtained from the food industry, where it is available from the manufacturers of diet soups. 'Gistex', salt-free powder contains less than 1% NaCl, is 8 times cheaper than the yeast extract from Difco Laboratories, but is as good or even slightly better in supporting nematode growth (Table I, media 1 and 2). Yet it will not substitute effectively for the soy-peptone portion of the medium (Table I, media 5, 6 and 7). Culture

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[8] The term haemiglobin is more correct because this protein is currently available in its oxidized state only. Haemoglobin is maintained here because it is more common. The actual state of iron in the heam group (Fe^{2+} or Fe^{3+}) does not affect nematode growth.

Table I. Growth of *Caenorhabditis elegans* in various media

Media	0-Day population (nem/ml)	7-Day population (nem/ml)	14-Day population (nem/ml)	Generation time (days ± SE)
1 Difco YE (3%), SP (3%), HLE (10%)	2,000	43,000	103,000	5.4 ± 0.2
2 Gistex (3%), SP (3%), HLE (10%)	2,100	53,000	96,000	4.1 ± 0.1
3 Gistex (3%), SP (3%), dextrose (1%), bacto-casitone (1%), haemoglobin (500 µg/ml)	2,000	50,000	128,000	4.6 ± 0.1
4 Gistex (3%), SP (3%), dextrose (1%), acid precipitated haemin chloride (50 µg/ml)	2,200	26,000	48,000	6.4 ± 0.3
5 Gistex (6%), dextrose (1%), bacto-casitone (1%), acid precipitated haemin chloride (50 µg/ml)	2,200	10,000	21,000	9.0 ± 0.4
6 Gistex (6%), dextrose (1%), acid precipitated haemin chloride (50 µg/ml)	2,000	6,400	12,600	7.5 to nm
7 Gistex (5%), dextrose (1%), acid precipitated haemin chloride (50 µg/ml)	2,250	5,500	8,400	9.5 to nm

Nematodes were grown in shallow liquid medium in screw-capped Erlenmeyer flasks (population growth) or culture tubes (determination of generation time with the larval assay method[10]). Population estimates are accurate at 10%. The pH of all media was 5.2. Nm, non-maturing; YE, yeast extract; SP, soy-peptone; HLE, heated liver extract.

Table II. Growth of *Caenorhabditis elegans* in suspension culture

Time (days)	1 l culture		10 l culture	
	Population number (nematodes/ml)	pH of medium	Population number (nematodes/ml)	pH of medium
0	3,750	5.20	8,800	5.51
2	7,900	5.29	15,000	5.49
4	18,500	5.60	26,000	5,61
7	76,000	6.50	90,000	6.07

medium consisting of 3% gistex, 3% soy-peptone, 1% dextrose, 1% bacto-casitone and 500 µg/ml haemoglobin (Merck, Federal Republic of Germany) was chosen for large scale cultivation of *Caenorhabditis elegans*.

BUECHER and HANSEN[9] first reported on the mass culturing of axenic nematodes in deep medium using continuous aeration. Cultures were performed in glass washing bottles. A 500 ml culture of *C. elegans* in a medium containing 3% yeast extract, 3% soy-peptone, 0.7% dextrose, MEM vitamins® 100 × solution (Grand Island Biological Co., Grand Island, New York) and HLE reportedly yielded a nematode population of over 100,000 nematodes per ml and a wet weight of 5 g of nematode tissue. The large scale cultivation technique reported here is applicable to quantities up to 10 l and over. It combines the effect of constant aeration and stirring in glass ware (spinner flasks) that has originally been designed for the axenic and semi-continuous culture of suspended cells.

A typical experiment is set up as follows. Nematodes are grown up in 100 ml of medium divided over five 250 ml screw-capped Erlenmeyer flasks. When the total yield is at least 2×10^6 nematodes, the cultures are transferred into a small spinner flask containing 1 l of culture medium including 1 ml of antifoam emulsion M-30 (Serva, Federal Republic of Germany). Antibiotics are routinely added to prevent contamination: penicillin G at 1,000 U/ml, streptomycin at 1 mg/ml and fungizone (amphotericin B) at 10 µg/ml. Sufficient air flow is bubbled through the medium, which is mixed slowly by a magnetically driven stirrer mechanism. The air is sterilized first by passage through a 1 inch in-line filter holder equipped with a 0.22 µm pore size membrane. When the population is near its maximum, i.e. when at the end of the exponential growth phase the population growth rate at first slows down, it is transferred into fresh medium in a 10 l capacity spinner flask and grown up until harvest. As with thin film cultures, the pH of the medium rises constantly with increasing nematode number and thus must be frequently checked. Any rise above pH 6.5 is readily followed by subsequent death of a significant part of the nematode population. Fatal rise of the pH can be overcome by the use of moderately acid medium (e.g. pH 5.2 with acetic acid) and by acidifying as needed, or by passage of the air through an acetic acid solution prior to bubbling through the medium.

Population growth of *C. elegans* and coincident changes of the pH of the medium are represented in Table II. It was not attempted to correct for the rise in pH in the 1 l culture; but in the 10 l culture the air was bubbled first through a 25% solution of acetic acid. The total number of nematodes obtained from the 10 l culture was near 1 billion. These were harvested and washed 3 times in 45% (w/w) sucrose solution yielding a wet weight of almost 40 g of very clean nematode tissue. This was obtained in less than 6 weeks, starting from some 50 worms.

This is the first report, to my knowledge, of the cultivation of nematodes in conditions and with yields that are remarkably comparable with those reported for cells and bacteria. There is probably no restriction to further cultivation of suitable free-living nematodes in large capacity fermentators.

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Continuous Labelling Method for Autoradiographic Analysis of Cell Cycle Parameters in Steady State Cell Systems[1]

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Summary. Under certain conditions, continuous availability of ^{3}H-thymidine in steady-state cell systems results in a linear increase of the fraction of labelled cells, the equation of which can be used to determine cell cycle parameters of the system investigated.

For analysis of cell cycle parameters in cell renewal systems, a method introduced by QUASTLER and SHERMAN[2] is usually employed. The method is based on flash labelling of DNA-synthesizing cells by ^{3}H-thymidine (^{3}H-TdR) and subsequent autoradiographic observation of the percentage of labelled mitoses (PLM-method). If wavelike oscillations of labelled mitoses are observed, the experiment gives information about the duration of all phases of the cell cycle. However, if clearcut second waves of labelled mitoses are not obtained, due to strong variations in cell cycle parameters, the available information may be limited to the premitotic gap (t_{g_2}), the mitotic time (t_m) and DNA-synthesis time (t_s), while the cell cycle time (t_c) cannot be measured. Serious problems may arise from early reutilization of ^{3}H-TdR labelled DNA in the cell system under investigation, as demonstrated for granulocytopoiesis[3]. In this situation, a cohort of flash-labelled cells cannot be produced, waves of labelled mitoses are not observed and the method fails to give any results. In order

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